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**Use of biomarkers in zebrafish as a
tool to evaluate the impact of
endocrine disruptors in aquatic
ecosystems**

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Index

1. Introduction	7
1.1 Contaminants of Emerging Concern: Occurrence and Fate	7
1.1.1 Flame Retardants: Properties and Fate.....	8
1.1.2 OPFR Occurrence in the Aquatic Environment	13
1.1.3 Presence in Biological Organisms	18
1.2 Toxicity: Flame retardants, Compounds with Endocrine Disruption	20
1.2.1 Zebrafish as a model for Environmental Toxicology	21
1.2.2 Transcriptomics as a Tool for Analysing Toxicity	22
1.3 A literature review of TBOEP Genes Alteration	22
2. Objectives	24
3. Methodology	25
3.1 Evaluated Compound: TBOEP	25
3.2 Ecotoxicology Assay with Zebrafish	26
3.2.1 Breeding and Dose-Response Assay	26
3.2.2 Sample Preparation for Transcriptomic Analysis	27
3.2.3 Transcriptomic Analysis by Quantitative Real-Time PCR.....	28
3.2.4 Quantification of qRT-PCR Results	31
4. Results and Discussion	32
4.1 Survival and Swim Bladder Inflation Rate	32
4.2 Effects of TBOEP at the Transcriptomic Level	32
4.3 Environmental Implications in Aquatic Ecosystems	36
5. Conclusions	37
6. References	38
7. Supplementary Data.....	44
7.1 Literature Review of Genes Altered and their Respective Fold Change	44
7.3 RNA Concentration After Extraction and Purity Index	46
7.4 Fugacity model's methodology.....	47

Abbreviations

TBOEP	Tris (2-butoxyethyl) phosphate
OPFR	Organophosphate Flame Retardant
OPE	Organophosphates esters
RW	Raw Water
FW	Filtered Water
UW	UV-Water
DW	Drinking Water
WWTP	Waste Water Treatment Plant
DWTP	Drinking Water Treatment Plant
hpf	Hours post fertilization
dpf	Days post fertilization
PBDE	Polybrominated diphenyl esters
qRT-PCR	Quantitative Real-Time PCR
CEC	Contaminants of Emerging Concern
K_{ow}	Octanol-Water Constant
BCF	Bioaccumulation Factor
K_H	Henry's Constant

Abstract

Wastewater regeneration has been recognized as an effective strategy to overcome water scarcity. To ensure quality, water must be treated to reduce its contaminants. However, the efficiency in removing contaminants at low concentrations is not high enough, and some persist in the treatment effluent. Among these contaminants are flame retardants, which are classified into aryl, alkyl, and chlorinated types. This study examined the physicochemical properties of 12 flame retardants to understand the variation in their concentrations in the environment and the potential risk each compound may pose.

To evaluate the toxicological effect of organophosphate flame retardants, the compound with the highest concentration in water was selected for toxicogenomic analysis. To evaluate the impact of the compound, tris(2-butoxyethyl) phosphate (TBOEP), a bioassay was performed using zebrafish larvae exposed to various concentrations of TBOEP (8.23, 28.77, 100.6, 352, and 1230 $\mu\text{g/L}$) to assess the transcriptomic changes that the compound could cause in the organism.

From the literature, it has been observed that TBOEP acts as an endocrine disruptor, affecting developmental processes, oxidative stress, DNA methylation, and presenting neurotoxic effects. In this study, the results of the transcriptomic analyses show that one of the studied genes, *cyp2k18*, tends to be deregulated at all doses. This trend suggests that TBOEP is a xenobiotic compound for the organism, activating detoxification pathways.

Resum

La regeneració d'aigües residuals ha estat reconeguda com una estratègia efectiva per combatre l'escassetat d'aigua. Per garantir-ne la qualitat, l'aigua ha de ser tractada per reduir els compostos químics presents. Tanmateix, l'eficiència en l'eliminació de contaminants a baixa concentració no és prou elevada, i alguns persisteixen en l'efluent del tractament. Entre aquests contaminants es troben els retardants de flama, classificats en arílics, alquílics i clorats. En aquest treball, s'han estudiat les propietats fisicoquímiques de 12 retardants de flama amb l'objectiu d'entendre la variació en les seves concentracions al medi i el risc potencial que pot generar cada compost.

Posteriorment, per tal de contribuir a definir l'efecte toxicològic dels retardants de flama organofosforats, es va seleccionar el compost amb major concentració a les aigües per dur a terme una anàlisi toxicogenòmica. Per avaluar l'impacte del compost, el tris (2-butoxi)etil) fosfat, (TBOEP), es va realitzar un bioassaig amb larves de peix zebra que van ser exposades a diverses concentracions de TBOEP (8,23, 28,77, 100,6, 352 i 1230 µg/L) amb l'objectiu d'avaluar l'alteració a nivell transcriptòmic que el compost podria provocar a l'organisme.

A nivell bibliogràfic, s'ha observat que el TBOEP actua com a disruptor endocrí, afectant processos de desenvolupament, estrès oxidatiu, metilació de l'ADN i presentant efectes neurotòxics. En aquest treball, els resultats de les anàlisis transcriptòmiques mostren que un dels gens estudiats, el *cyp2k18*, presenta una tendència a la desregulació en totes les dosis. Aquesta tendència suggereix que el TBOEP és un compost xenobiòtic per a l'organisme, activant les vies de desintoxicació.

1. Introduction

1.1 Contaminants of Emerging Concern: Occurrence and Fate

The urban water cycle consists of distinct parts: irrigation water, industrial use, household consumption, wastewater treatment plants (WWTPs), and water purification plants (*Figure 1*). Water is transported across the territory via rivers or aquifers until it flows into the sea. There are pollution inputs at various points in this water network, with concentrations ranging from mg/L to ng/L.



Figure 1 Water management cycle. Source: Aqualia

These sources of pollution have different origins, either industrial or agricultural. Nitrogen, phosphorus, and ammonia are the compounds present in the highest concentrations and are the main focus of pollution in the aquatic environment, ranging in concentrations of mg/L. Consequently, WWTPs were established to reduce these concentrations. However, other types of contamination derive from human activities, and in most cases, come from a synthetic origin, generated to meet the needs of everyday human activities in the last centuries, and they are called Contaminants of Emerging Contaminants (CECs).

CECs include various contaminants, such as pharmaceuticals, personal care products, pesticides, food additives, microplastics, engineered nanomaterials, and their metabolites and transformation products. Examples as ibuprofen, nicotine, codeine, diazepam, and plasticizer additives, like flame retardants. These contaminants have not been prioritized to be monitored in water samples due to their low concentrations, ranging from ng/L to $\mu\text{g/L}$. The effect that these compounds generate in organisms is still unknown nor which risks are associated with environmentally relevant concentrations (Nikolenko et al., 2023).

With the growing demand for clean freshwater and the concurrent rise in CEC occurrence in European groundwater bodies, it is crucial to assess whether CECs might limit the potential use of urban groundwater as a drinking water source (Nikolenko et al., 2023). To answer this question, it is key to understand the methods of action of all groups of chemicals found in water samples. **Flame retardants, as a group, have a research gap regarding their effects, and further studies should be developed to address this** (Pantelaki & Voutsas, 2019).

1.1.1 Flame Retardants: Properties and Fate

Flame retardants are synthetic chemical additives incorporated into flammable materials to prevent or delay the onset of combustion. Their use is crucial for preventing damage and loss, both material and human. Consequently, flame retardants are found in a variety of products, including electronics, vehicles, polyurethane foams, and textiles (Pantelaki & Voutsas, 2019). Most flame retardants operate primarily in the solid phase of burning materials by promoting char formation, although some also function in the gas phase. They can be chemically bonded to a polymer or simply mixed into it. Chemically bonded retardants experience limited losses during the product's lifetime, whereas the concentration of mixed retardants may decrease over time.

Flame retardants have been used since the 1960s. Historically, polybrominated diphenyl ethers (PBDEs) were commonly used. However, due to their toxicity and bioaccumulation in food chains, they were classified as Persistent Organic Pollutants at the Stockholm Convention in 2008 and consequently eliminated in the EU and the US (Pantelaki & Voutsas, 2019). Despite this, the need for flame retardants persisted, leading to the rise of emerging compounds such as organophosphates (OPFRs) and new brominated flame retardants (NBFRs). These substitutes seemed a good replacement due to their vapor-phase activity and similar chemical properties to PBDEs. As a result, OPFRs have become high-volume production chemicals, exceeding 1000 tonnes per year in Europe, and in a global scale was 1.05 million tonnes in 2018 (Yang et al., 2022).

At *Figure 2*, there are described the types of flame retardants and their proportion of use in 2019. Organophosphate flame retardants correspond to 18% of the global consumption of flame retardants.

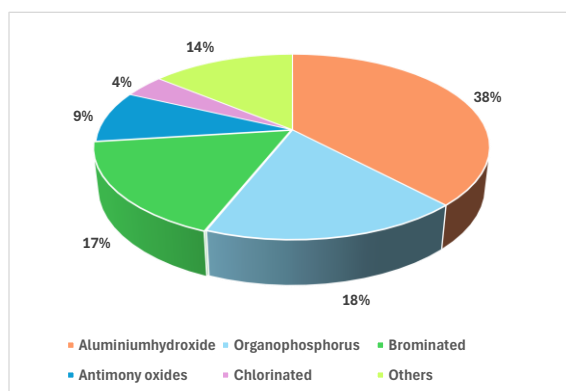


Figure 2 Industry estimation of total consumption of flame retardant in Europe (Regulatory Strategy for Flame Retardants, 2023)

Organophosphates consist of a diverse class of compounds characterized by their phosphorus-containing structures. While sharing this common feature, their specific chemical structures and resulting properties vary widely, enabling their uses for several functions (e. g. pesticides, plasticizers and flame retardancy).

OPFRs can be categorized into three main groups, phosphinates, phosphonates and phosphate esters (OPE).

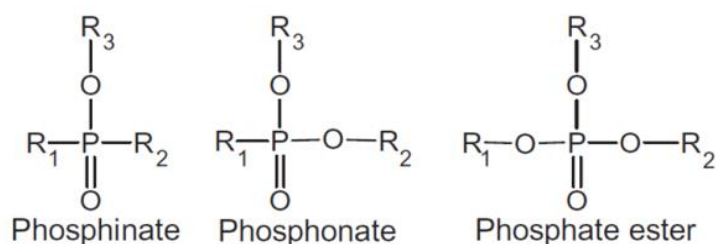


Figure 3 Groups of OPFRs (van der Veen & de Boer, 2012)

The three groups have in common a pattern that every oxygen is united to a phosphate group by an alkaline chain or a benzene ring (Yang et al., 2022). The main difference between them is that phosphinates include inorganic OPFRs, whereas phosphonates consist of organic and halogenated OPFRs, and phosphate esters have an intern classification between chlorinated, alkaline and aryl (van der Veen & de Boer, 2012) (Hou et al., 2016a).

Phosphinates are used in polyamides, polyesters and other polymers for their high thermal stability and effective flame retardancy at low loadings. Phosphonates are used in polyurethanes, epoxy resins, and thermoplastics due to their versatile flame-retardant properties and ability to improve mechanical properties. Phosphate esters are used in PVC, polyurethane foams, and engineering plastics, often where additional plasticizing properties are beneficial.

In this project, I focused on phosphate esters and how flame retardants' way of action differs between each class because of their chemical properties. To conduct a more in-depth study of the differences between the types of OPFRs, several examples of each type of organophosphate are presented in the following table (Table 1), which includes principal factors in assessing the behaviour of OPFRs in the environment and for assessing their influence on organisms.

Chemical properties considered were molecular mass, vapor pressure, Henry's constant, water solubility, the bioaccumulation factor (BCF), and octanol-water constant's logarithm ($\log K_{ow}$) which it is used to predict the partitioning behaviour of a chemical between lipid and water phase.

Henry's constant (K_H) is a proportionality factor that describes the solubility of a gas in a liquid at a constant temperature. It allows to understand the correlation between the amount of flame retardants that erode into the air and their subsequent pollution on it, in the aquatic environment (Pantelaki & Voutsas, 2019). Higher Henry's law constant compounds are more likely to move from the air into water bodies. Vapor pressure determines how easily flame retardants can evaporate into the air from a liquid or solid phase. Higher vapor pressure indicates greater volatility, which can influence the extent to which a chemical is released into the atmosphere. On the other hand, aquatic organisms, such as zebrafish, rely on water as their medium for respiration, which means that the more soluble a compound is, the more likely the organisms will be exposed to them.

While OPFRs studied exhibit a positive $\log K_{ow}$, indicating their inherent lipophilic nature, their K_{ow} range (4.3 to 9.9) suggests they generally have lower affinity for lipid content in fishes compared to polybrominated diphenyl esters, the preceding flame retardants commonly used, whose range is much wider (from 129 to 33,430), highlighting differences in their potential for bioaccumulation in aquatic organisms.

Table 1 A representation of several organophosphate esters and their chemical nomenclature and properties: Molecular Weight, Chemical Formula, Henry's Law Constant, Vapor Pressure, Water Solubility, Octanol-water Constant's logarithm, and the Bioaccumulation Factor (Pantelaki & Voutsas, 2019).

Compound	Abbreviation	Type of OPE	CAS n°	Molecular Weight	Chemical formula	K_H (atm m ³ mol ⁻¹)	V_p (mmHg, 25 °C)	S (mg L ⁻¹ , 25 °C)	log K_{ow}	BCF
Tris (chloroethyl) phosphate	TCEP	Chlorined	115-96-8	285.49	C ₆ H ₁₂ Cl ₃ O ₄ P	1.67E-07	6.10E-02	7000	1.63	0.425
Tris (2-chloroisopropyl) phosphate	TCIPP	Chlorined	13674-84-5	327.57	C ₉ H ₁₈ Cl ₃ O ₄ P	4.69E-07	5.64E-05	1200	2.89	3.27
Tris (1,3-dichloro-2-propyl) phosphate	TDCIPP	Chlorined	13764-87-8	431.1	C ₉ H ₁₅ Cl ₆ O ₄ P	1.08E-07	2.98E-07	7	3.65	21.4
Trimethyl phosphate	TMP	Alkyl	512-56-1	140.08	C ₃ H ₉ O ₄ P	2.57E-07	8.50E-01	50000	-0.6	3.16
Triethyl phosphate	TEP	Alkyl	78-40-0	182.15	C ₆ H ₁₅ O ₄ P	3.55E-06	3.90E-01	50000	0.87	3.16
Tripropyl phosphate	TPP	Alkyl	513-08-06	224.23	C ₉ H ₂₁ O ₄ P	8.25E-06	2.40E-02	6450	2.35	0.912
Tris (isobutyl) phosphate	TIBP	Alkyl	126-71-6	266.31	C ₁₂ H ₂₇ O ₄ P	2.76E-04	1.30E-02	475.6	3.6	19.51
Tris (2-butoxyethyl) phosphate	TBOEP	Alkyl	78-51-3	398.47	C ₁₈ H ₃₉ O ₇ P	3.28E-07	1.23E-06	1100	3	25.56
Tri-n-butyl phosphate	TNBP	Alkyl	126-73-8	266.31	C ₁₂ H ₂₇ O ₄ P	1.66E-04	1.31E-01	280	3.82	39.81
Tris (phenyl) phosphate	TPHP	Aryl	115-86-6	326.28	C ₁₈ H ₁₅ O ₄ P	1.96E-07	1.12E-05	4.7	4.7	113.3
2-Ethylhexyl diphenyl phosphate	EHDPP	Aryl	1241-94-7	362.41	C ₂₀ H ₂₇ O ₄ P	1.96E-07	5.00E-05	1.9	6.3	855.3
Tris (methylphenyl) phosphate	TMPP	Aryl	1330-78-5	368.36	C ₂₁ H ₂₁ O ₄ P	9.21E-07	1.10E-07	0.3	6.34	2534

In *Figure 4*, the correlations between several properties from *Table 1* are graphed. In *Figure 4a*, a linear correlation is observed between the logarithm of the Bioaccumulation Factor ($\log BCF$) and the logarithm of the Octanol-Water Partition Coefficient ($\log K_{ow}$), indicating that compounds with a higher tendency towards the lipophilic phase are more likely to remain within organisms. *Figure 4b* demonstrates that $\log K_{ow}$ decreases as a compound's water solubility increases. In *Figure 4c*, the relationship between $\log K_{ow}$ and Molecular Weight is shown for various compounds.

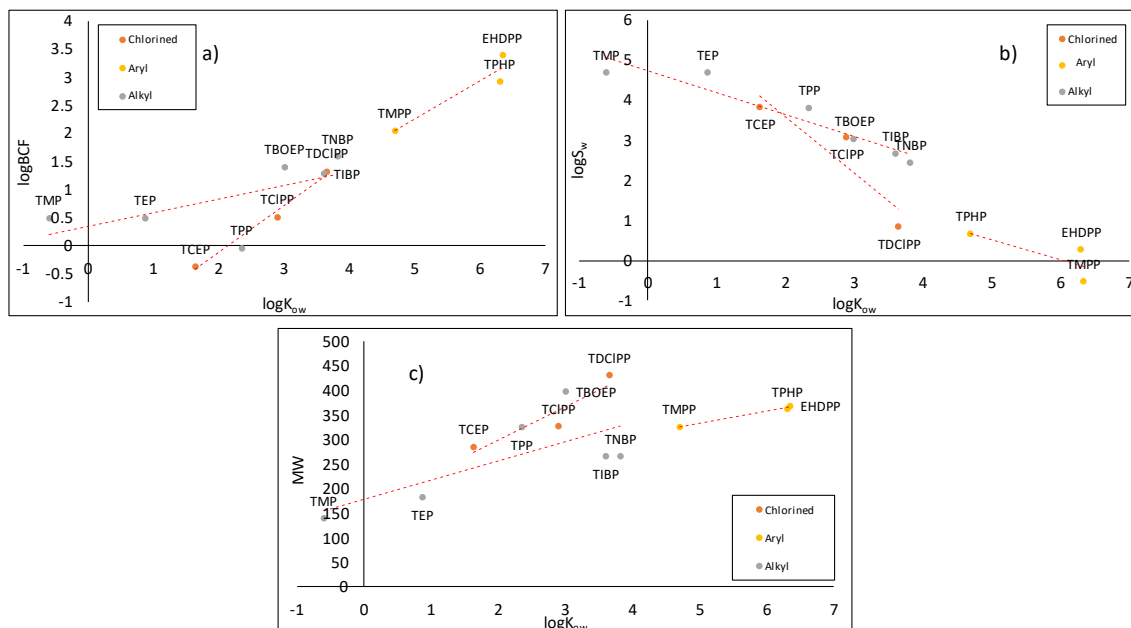


Figure 4 a) Correlation between $\log BCF$ and $\log K_{ow}$; b) Correlation between \log Water Solubility and $\log K_{ow}$; c) Correlation between Molecular Weight and $\log K_{ow}$, Own Source.

In comparison to chlorinated and aryl OPFRs, alkyl OPFRs have a lower molecular weight, but exhibit similar bioaccumulation factors and water solubility as chlorinated OPFRs. Aryl OPFRs, on the other hand, demonstrate low volatility and water solubility while exhibiting the highest hydrophobicity and bioaccumulation potential among the three types. Both aryl and alkyl-OPFRs show a higher affinity for sediment and soil (van der Veen & de Boer, 2012).

Despite these characteristics, compounds within the OPFR family -particularly chlorinated and aryl OPFRs- exhibit high stability and are easily released into aquatic environments. This leads to their persistence and propensity for widespread bioaccumulation, in hand with the problem that some OPFRs have been confirmed to generate chronic toxicity and neurotoxicity in aquatic organisms.

1.1.2 OPFR Occurrence in the Aquatic Environment

Atmospheric washout from precipitation and industrial discharge from factories and wastewater treatment plants (WWTPs) are the primary ways that OPFRs enter aquatic and terrestrial systems (Cristale et al., 2013; Loos et al., 2012; Marklund et al., 2005). In aquatic environments, OPFR are present in WWTP effluents and sludge, sediment, and surface water (Behnami et al., 2024). Both treated and untreated wastewater discharges are considered significant pathways for OPFRs entering surface waters, potentially impacting groundwater through infiltration (Pantelaki & Voutsas, 2019).

To further study the concentration distribution in the aquatic environment, it is key to consider the organophosphate flame-retardant per capita mass load into wastewater. As an example, *Figure 5* shows OPFR input into WWTP from Australia, where TBOEP is the compound with the highest per capita input, followed by TCIPP in all cases, and TCEP and TNBP (O'Brien et al., 2015).

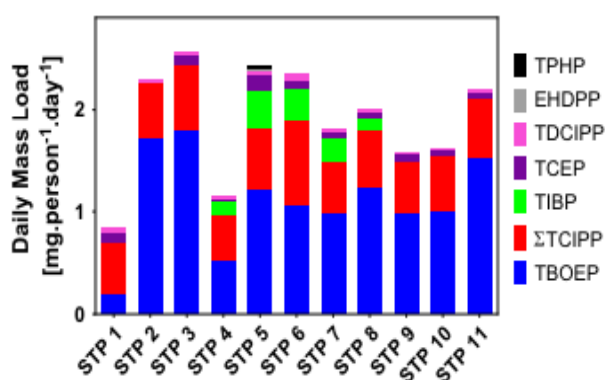


Figure 5 Daily per capita input of OPFR mass loads into eleven wastewater treatment plants in Australia.

Figure from O'Brien et al., 2015.

Pantelaki & Voutsas, 2019 reviewed ranges of OPFRs concentration in effluent wastewater, surface water and sediments in several countries reported in the literature, which are summarized in *Figure 6*. They showed that Canada is the country where TBOEP has almost double the concentration than in Spain or Austria in effluent wastewater (*Figure 6a*). For TCPP, there is no such difference between Canada and Spain, it follows the same pattern for different concentration compounds with different absolute values. However, related to Austria, despite having the same two maximum concentration compounds, the rest of the concentrations do not follow the same structure, the concentration of TDCPP and TCEP have values as high as TCPP, which is not observed in the other countries.

Figure 6c shows that, in Germany, flame retardants in surface water are found at low concentrations. For Spain, TBOEP and TCPP are the only compounds with relevant concentrations, which is in concordance with the highest concentrations found in wastewater effluents. Moreover, comparing concentrations found in wastewater effluents and in surface waters, it can be observed that the concentrations of flame retardants are conserved through the water flow, since from the moment it gets out of the WWTP, the concentrations do not fluctuate.

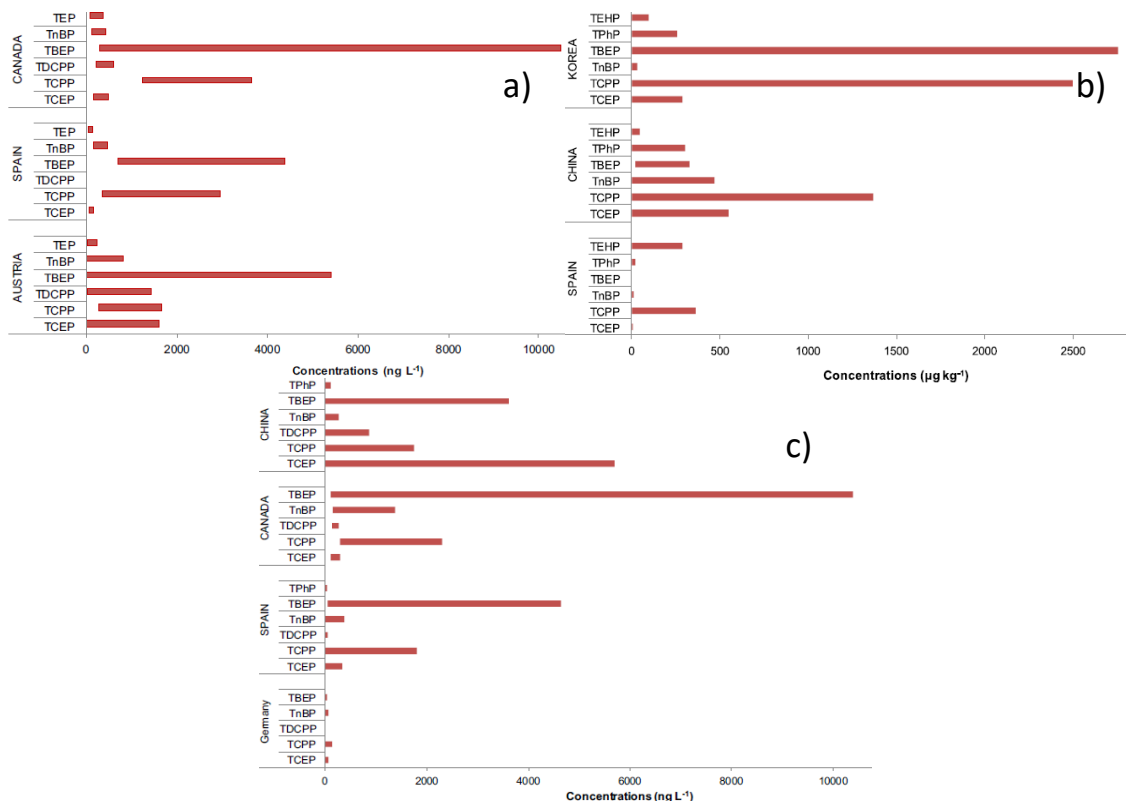


Figure 6. Range of OPFR's concentration in a) effluent wastewater, b) sediments; c) surface water reported in the literature. Figures from Pantelaki & Voutsas, 2019

Taking all the exposed above, we can conclude that TBOEP and TCIIP are the two main compounds found in wastewater effluent, surface water, and sediment, and they persist throughout the process, since the mass load input in WWTP until its effluent, a situation that can be justified with the fact that flame retardants are not well eroded from wastewater treatment plants.

1.1.2.1 Flame Retardant Removal Efficiency from Wastewater Treatment Plants

Wastewater treatment plants are vital for controlling the release of all types of contaminants into the environment (Lopez et al., 2022). These plants typically use a conventional treatment process that includes primary sedimentation, biological treatment, and secondary sedimentation.

While these processes effectively remove biodegradable carbon, nitrogen, phosphorus, and pathogens, necessary equipment is lacking to manage complex pharmaceuticals, pesticides, and personal care products. Consequently, WWTPs become significant sources of flame retardants, along with their metabolites and transformation products (Struzina et al., 2024)(Lopez et al., 2022).

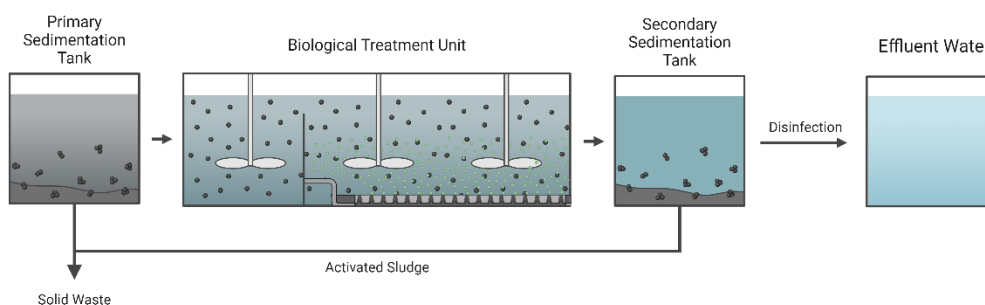


Figure 7 Scheme of conventional WWTPs process: a primary sedimentation tank, the biological treatment unit, and secondary treatment which leads the effluent to the aquatic environment. Source: Biorender

As it is shown in Figure 7, the primary sedimentation stage involves the removal of solid particles through gravity. However, many flame retardants remain in the aqueous phase due to their low sorption coefficients, making them less likely to settle out during this process (Lopez et al., 2022). In secondary treatment, biological processes can degrade some organic contaminants; however, they are resistant to biodegradation. During secondary sedimentation, additional suspended solids are removed, but their solubility in water complicates the process. Chlorinated OPFRs, such as TCIPP, are considered resistant to degradation and often detected in WWTP effluents (Marklund et al., 2005). For example, O'Brien et al., 2015 studied that TCIPP represents 69% of annual load of OPFRs discharged to the environment from all WWTP in Australia. One alkyl compound, TBOEP, concentrations is significantly reduced through biological treatment, but not eliminated, (e.g. in the influent is 3292 ng/L, and it is reduced to 581 ng/L), indicating partial degradation or transformation (Woudneh et al., 2015).

To meet higher purity standards for effluents discharged into groundwater, tertiary treatments like coagulation-flocculation, sand filtration, and UV disinfection are employed (Zhang et al., 2023). However, the reduction of concentration in aquatic treatment lines doesn't mean that flame retardants are eliminated from the system, another issue arises, which is located in the solid waste stream, because flame retardant concentration increases, as can be observed for TBOEP, where its concentration in activated sludge increased from 1420 $\mu\text{g}/\text{kg}$ to 2236 $\mu\text{g}/\text{kg dw}$ (Woudneh et al., 2015).

Once released into the environment, the fate of flame retardants is influenced by their chemical properties and environmental conditions. In surface waters and groundwater, these compounds undergo various transport and chemical processes. Microbial degradation is considered the primary attenuation mechanism in aquifers, as adsorption tends to be reversible and retards the transport rather than removing the contaminants (Nikolenko et al., 2023). Within the territorial context of Catalonia, in a study by Nikolenko et al., 2023 of the Besòs River's aquifers, which are recharged by WWTP effluent water, there were identified high concentrations and a wide variety of contaminants of emerging concern, including flame retardants. This would suggest that microbial degradation is not efficient enough to manage the water input. Therefore, alternative strategies must be considered to improve water quality.

1.1.2.2 Flame Retardant Removal Efficiency from Drinking Water Treatment Plants

Drinking Water Treatment Plants (DWTP) aim to provide safe drinking water by removing contaminants, but the presence of OPFRs poses significant challenges. The resistance to biodegradation, apart from the technical limitations of plants, is due to its chemical stability and its sorption characteristics. OPFRs tend to remain in the aqueous phase, rather than binding to solid particles. Therefore, coagulation and flocculation, sedimentation, and filtration processes, shown in *Figure 8*, which are commonly used in DWTP, are inefficient for these compounds.

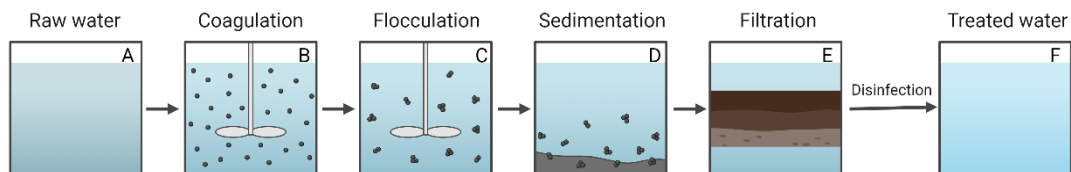


Figure 8 Schematic DWTP train. A - Raw Water (RW), B, C and D – Coagulation, Flocculation and Sedimentation, E - Filtered Water (FW), which is UV disinfected water (UW), F – Treated water. Source: Biorender

While activated carbon filtration can partially remove OPFRs, its efficiency varies. Advanced oxidation processes like ozonation and UV/H₂O₂ degrade OPFRs, but complete mineralization is not always achieved. Membrane filtration methods like reverse osmosis and nanofiltration effectively remove OPFRs, but they are energy-intensive and costly. In drinking water treatment facilities, the total average concentration of a sample of fifteen organophosphate esters was 501 ng/L in raw water and 162 ng/L in treated drinking water, resulting in an average removal efficiency of 67%.

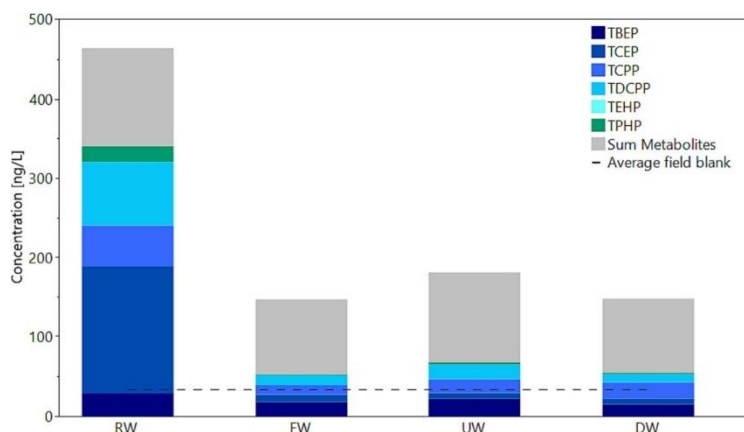


Figure 9 Organophosphate esters concentrations throughout DWTP (Struzina et al., 2024).

As can be observed in Figure 9, TBOEP is the compound that rests constantly in all phases of the treatment. It is key to mention that TCEP, TPHP, and TDCIPP have high efficacy of removal (94.0, 86.5, and 90.7%), however, TBOEP has 56.7% and TCIPP 56.5%. Therefore, this is consistent with the fact that these two compounds are more frequently found in surface water samples, as previously mentioned in Figure 6. It can be concluded the urgent need for advanced water treatment technologies and regular water quality monitoring to address these challenges effectively (Struzina et al., 2024).

1.1.3 Presence in Biological Organisms

The food web is a complex network of feeding relationships among organisms in an ecosystem. It demonstrates how distinct species are interconnected through their dietary preferences and energy flow. There are producers, primary consumers, secondary consumers, and apex predators, where each level has its role.

Biomagnification is the capacity of a compound to be transferred in the food web, which depends on the physicochemical properties of the chemical and the metabolic transformations produced by organisms. In benthic food webs, the biomagnification factors (BMFs) for TBOEP, TCIPP, and TCEP are less than 1, 3.5, and 2.6, respectively (Brandsma et al., 2015).

Despite the low BCF compared to brominated flame retardants, Brandsma et al., 2015 studied the accumulation of OPFRs in several species and compounds in the Benthic and Pelagic Food Web. As illustrated in *Figure 10*, the most detected retardants were TBOEP, TCIPP, and TIBP, which correspond to the highest concentrations found in water samples. This trend highlights the relationship between the availability of these chemicals for ingestion by organisms and the total amount of OPFR produced.

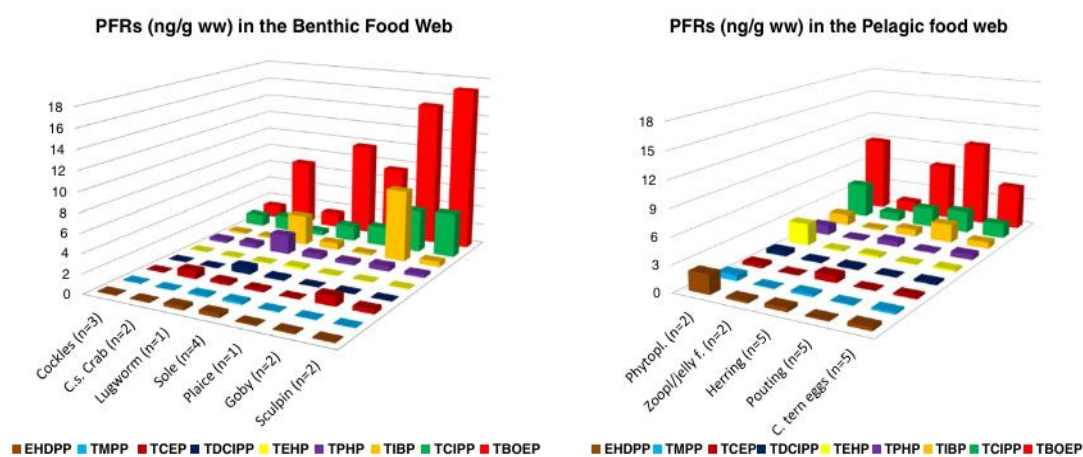


Figure 10 Median OPFR levels in ng/g in benthic and pelagic organisms (Brandsma et al., 2015)

Other studies, also verify *Figure 10's* results, because TCIPP, with a BCF of 8.51, was found at relatively elevated levels in rainbow trout from Lake Ontario (Bestvater, 2014) and also in mussels from lakes and coastal areas in Sweden (Marklund Sundkvist et al., 2010).

1.1.3.1 Fugacity Model Comparing the distribution of OPFRs in Water, Fish and Their Diet

To create a deeper study, a fugacity model was developed using the compounds listed in Table 1. The methodology used is summarized in the *supplementary data*. Several assumptions were made to simplify the calculations. The first assumption was that the half-life time of all compounds is 10h, based on the value calculated by Wang & Song, 2024 for TDCPP. The concentration in water was set at 0,2 ng/L for all compounds, representing a low dose, as shown in *Figure 6 b*). For the concentration of fish ingestion, 25 µg/L was assumed, as it is one of the exposure doses selected for the experiments in this project.

Related to the risk of bioaccumulation in the aquatic environment, aryl compounds are the ones with less fugacity when are compared to alkyl and chlorinated. Despite their high K_{ow} and BCF, the almost non-solubility in water of these compounds resulted in almost non-presence in the aquatic environment. On the other hand, alkyl flame retardants are very water-soluble, and they maintain a relatively high K_{ow} , therefore, the quantity available and fugacity for fish's diet increase.

Table 2 Fugacity model for OPE studied in fish, its diet and, in water. Each value is multiplied per 10^{-10} .

Compound	Type	Fugacity		
		Water	Fish's diet	Fish
TCEP	Chlorined	0.12	6947.24	3.08
TCIPP		0.29	934.45	7.51
TDCIPP		0.05	28.41	1.29
TMP	Alkyl	0.37	3700356.84	9.67
TEP		3.95	1331943.47	102.68
TPP		126.32	47797.73	3169.12
TIBP		210.02	131888.89	5343.61
TBOEP		0.17	417.03	4.31
TNBP		7.46	83262.00	193.62
TPHP	Aryl	0.12	6.07	2.48
EHDPP		0.11	0.14	0.24
TMPP		0.51	0.58	1.01

Results in *Table 2* show that aryl compounds bioaccumulate and biomagnify over the trophic chain because its value of fugacity in fish is higher than in its diet and water. In alkyl compounds, pollutants have a higher capacity of fugacity in fish's diet than in water or larger organisms because their capacity to be retained in the lipid phase is low. However, these results are insufficient for a comprehensive understanding of OPFR biomagnification in ecosystems, necessitating further investigations.

1.2 Toxicity: Flame retardants, Compounds with Endocrine Disruption

Toxic substances can affect biological organisms in several ways, primarily categorized as lethal or non-lethal effects. Lethal effects are dangerous, but non-lethal doses can produce complex and less predictable effects, often not immediately visible. The range of alterations is wide, from endocrine disruption to hormonal imbalances, neurotoxicity, carcinogenicity, and metabolic effects. They can appear in numerous ways, including behavioural changes, reduced growth, reproductive issues like reduced fertility, and altered reproductive timing. Also suppressing immune function may result in cognitive impairments, motor dysfunction, and oxidative stress which can lead to cell damage, inflammation, and accelerated aging. Understanding these diverse impacts is essential for fully assessing the risks toxic substances pose to organisms (Struzina et al., 2024).

Considering the prevalence of OPFRs in the environment, significant attention has been devoted to their possible adverse effects. OPFRs are identified as endocrine-disrupting chemicals that can mimic or block hormones, disrupting normal bodily functions by binding to hormone receptors, altering hormone synthesis, and affecting hormone metabolism. OPFRs have been found to interfere with the thyroid hormone pathway, crucial for brain development and metabolism regulation (Han et al., 2022)(Yan et al., 2022). Toxicological studies, not only associate flame retardants with endocrine disruption, but also that long-term exposure to OPFRs can cause reproductive and systemic effects in animals (van der Veen & de Boer, 2012). Epidemiological studies have linked OPFR exposure to developmental and reproductive issues in humans, such as lower birth weights and developmental delays in infants (X. Cheng et al., 2024). Aryl-OPFRs have been associated to induce heart toxicity by interfering with transcriptional regulators in zebrafish (Du et al., 2015).

Since 1998, the World Health Organization has suspected that tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) and, nowadays it is confirmed to enter the bloodstream and induce tumors in the liver, kidney, and testis (Hou et al., 2016b). Concerning TCEP, its use has been gradually restricted in Europe and North America, owing to its carcinogenic potential and neurotoxicity, leading to its replacement by TCIIP in commercial products (Y. Xu et al., 2022).

1.2.1 Zebrafish as a model for Environmental Toxicology

Zebrafish (*Danio rerio*), belongs to the family *Cyprinidae*, order *Cypriniformes*, and class *Actinopterygii*, characterized by ray-finned fins and a hinged jaw. This tropical teleost fish originates from Southeast Asia. Zebrafish are used in research due to their well-known genome, which shares 70% similarity with the human genome and includes over 84% of genes related to human diseases. Advantages for laboratory use include easy maintenance, rapid development, external fertilization, transparent embryos, low cost, and sexual maturity at three months, allowing multiple generations per year (Durán Hernán López-Schier, 2011; Zhao et al., 2024.)

Zebrafish are small, with adults typically measuring 3 to 5 cm in length and 1 cm in width. They have a fusiform shape, a dorsal fin, an upward-facing mouth, and 5 to 9 dark blue lateral stripes extending from behind the operculum to the end of the caudal fin. Males are golden with a slender body and gold anal fin, while females are silver with a rounder body, prominent abdomen, extended genital papilla, and silver anal fin (Singleman & Holtzman, 2014). Reproduction involves external fertilization, with ovulation triggered by male gonadal pheromones. Development is rapid, with organogenesis and angiogenesis occurring within 24 hours, and they reach adulthood at three months. The main embryonic stages include zygote (0 – ¾ hours post-fertilization (hpf)), cleavage (¾ - 2 ¼ hpf), blastula (2 ¼ - 5 ¼ hpf), gastrula (5 ¼ - 10 hpf), segmentation (10 – 24 hpf), pharyngula (24 – 48 hpf), hatching (48 – 72 hpf), and early embryo (>72 hpf, 3 days post-fertilization (dpf)) (Singleman & Holtzman, 2014).

The yolk sac, formed during segmentation, provides nutrients until exogenous feeding begins, it is a membranous sac attached to the embryo. Around 3 days post fertilization (dpf) embryos sporadically hatch and after they continue organ morphogenesis (except the gastrointestinal tract) and develop pectoral fins. Early embryos inflate their swim bladder, reposition their mouth, and develop intestinal and iridophore structures, enabling swimming and feeding movements (Singleman & Holtzman, 2014). According to Spanish Royal Decree 53/2013, *Danio rerio* is not considered a laboratory animal until five days post-fertilization (Núm, n.d.).

1.2.2 Transcriptomics as a Tool for Analysing Toxicity

Molecular biology is a tool that allows the prediction of broader biological and ecological impacts of stressors because allows the detection of early and subtle changes in organisms that may not be visible yet at the physiological or behavioural levels, being very useful for taking preventive measures before significant damage to population or ecosystems. These molecular changes are often manifested as alterations in the transcription of genes involved in key biological functions. By understanding which transcripts are altered, it is possible to understand which cellular processes are being disrupted (e.g. changes in transcript abundance of genes associated with neural development might predict neurotoxicity). Therefore, the identification of biomarkers of exposure and effects, which are specific genes that consistently respond to exposure to environmental contaminants, is key in environmental toxicological studies.

This mechanistic understanding is crucial for assessing the risks posed by contaminants to wildlife and human health. Furthermore, transcriptomic data contributes to robust risk assessments, aiding in the formulation of regulatory decisions and the development of effective environmental management strategies.

1.3 A literature review of TBOEP Genes Alteration

To understand the effects of flame retardants at the transcriptomic level, I conducted a literature review specifically on five studies involving zebrafish exposed to TBOEP (Ma et al., 2015; Jiang et al., 2018; Ma et al., 2016b; Liu et al., 2017; and Zeng et al., 2018). The supplementary data provide detailed tables containing all information recollected (*Table S1* and *S2*). However, the most relevant genes found are the summed-up ones in *Table 3*.

TBOEP exposure in zebrafish embryos has been shown to affect various nuclear hormone receptor pathways and disrupt neuroendocrine functions. Ma et al., 2015 found that exposure to 200 µg/L of TBOEP significantly upregulated estrogen receptor-associated genes, while genes related to the mineralocorticoid receptor pathway were downregulated. Other studies Ma et al., 2016b; Liu et al., 2017 and Jiang et al., 2018 investigated higher concentrations (up to 2500 µg/L) and reported developmental toxicity, including malformations, growth delay, reduced heart rate, and altered motor behaviour, likely due to gene expression changes in the GH/IGF axis. These molecular changes disrupted endocrine functions, leading to deformities and death. Zeng et al., 2018 at 100-10000 µg/L over extended exposure periods and found decreased body length and mass, along with

altered expression of GH/IGF and HPT axis genes. Bioaccumulation was higher in females, correlating with growth inhibition, reduced plasma thyroxine (T4), and decreased expression of thyroid-related genes, particularly at 60 days post-fertilization (dpf). These findings suggest that TBOEP impairs endocrine regulation and growth in zebrafish, with varying effects depending on concentration, duration, and developmental stage.

Table 3 Most relevant genes from the literature review

Pathway	Gene symbol
Involved in regulation of heart rate	<i>adrb2b</i>
Related to synthesis of testosterone	<i>17βhsd</i>
Involved in gamete generation	<i>ar</i>
Estrogen receptor	<i>er</i>
Predicted to be involved in cellular response to hormone stimulus and positive regulation of transcription by RNA polymerase II	<i>ncoa1</i>
Exhibits steroid binding activity and steroid hormone receptor activity	<i>pgr</i>
Response to xenobiotic, estrogen and estradiol stimulus	<i>vtg1</i>
Predicted to be structural constituent of myelin sheath	<i>mbp</i>
Nuclear receptor activity. Involved in regulation of transcription and thyroid	<i>thrb</i>
Responsible for iodine transport into the body and thyroid hormone production	<i>nis</i>
Involved in adipose tissue development and positive regulation of lipid catabolic process	<i>gh</i>
Involved in regulation of neural retina development	<i>deio1</i>
A protein precursor of thyroid hormone and can be used by the thyroid gland to produce the thyroid hormones T4 and T3	<i>tg</i>
Contribute to hormone activity	<i>tshb</i>
Predicted to have hormone activity and thyroid hormone binding activity	<i>ttr</i>
Predicted to have steroid hydroxylase activity. Involved in response to xenobiotic stimulus	<i>cyp3a65</i>
Mineralocorticoid receptor	<i>mr</i>
Exhibits insulin-like growth factor receptor binding activity. Involved in oocyte maturation and sperm capacitation	<i>igf3</i>
Cholesterol side-chain cleavage enzyme	<i>cyp11a</i>
Predicted to have hormone activity	<i>crh</i>
Involved in animal organ development	<i>shha</i>
Predicted to be involved in several processes, including negative regulation of cholesterol storage; positive regulation of fatty acid metabolic process; and regulation of transcription by RNA polymerase II	<i>ppara</i>
Predicted to have calmodulin binding activity and phospholipid binding activity. Involved in axon regeneration and tissue regeneration.	<i>gap43</i>

2. Objectives

The aim of this project is to characterize sublethal effects of TBOEP using zebrafish embryo bioassays exposed to environmentally relevant concentrations, to determine if the implementation of new purification technologies is necessary to ensure non-harmful levels in aquatic organisms.

To achieve this general objective, the following specific objectives were defined:

1. To contribute to acknowledging the effects of OPFRs.
2. To detect and quantify the range of concentrations, in acute exposure, where TBOEP provokes transcriptomic alterations in zebrafish larvae.
3. To identify which transcripts were altered in the presence of TBOEP.
4. To discuss the need to optimize the flame-retardant removal processes in wastewater treatment plants.

To achieve these specific objectives, the following tasks have been carried out:

1. Literature review on the concentrations of flame retardants found in the aquatic environment and wastewater treatment plants (Section 1).
2. Literature review on the effects of TBOEP in exposed zebrafish embryos and adults at the transcriptomic level (Section 1).
3. Study transcriptomic alterations in zebrafish embryos exposed to TBOEP from 4 to 5 days post fertilization and identify possible transcriptomic biomarkers of exposures (Section 3 and 4 of this document).

Given the objectives of this work and the literature review (Section 1), I hypothesize that the current levels of TBOEP in aquatic environments could be capable of causing transcriptomic alterations through the disruption of endocrine pathways in zebrafish embryos. I expect TBOEP to affect gene regulation, leading to noticeable changes in gene expression. Since this study looks at acute exposure, I think the effects will be less severe than those from long-term exposure but still significant enough to show potential harm. This suggests the need to review current wastewater treatment methods to reduce TBOEP levels and protect aquatic life.

3. Methodology

3.1 Evaluated Compound: TBOEP

For this project, the chosen compound to study is TBOEP (tris (2-butoxyethyl) phosphate) because, as shown in *Figure 6*, it is the most frequently found flame retardant in several countries, with high concentrations in the effluents of WWTPs, food webs, and water surfaces. TBOEP is a slightly yellow, oily liquid with chemical properties listed in Table 1. It is not only used as fire-resistant but also used as a light-stable plasticizer in the production of vinyl resins, rubber, nitrocellulose, and cellulose acetate, as well as synthetic rubber intended for food or drink (C₁₈H₃₉O₇P - PubChem). Its usage ranges between 5000 and 6000 tons per year. TBOEP is frequently detected in the environment; however, limited information is available regarding its toxicity and mechanisms of action.

To select the concentrations for the exposure, a literature review was conducted. A study on water samples in Austria found TBOEP's maximum concentration to be 5400 ng/L (Martínez-Carballo et al., 2007). To predict the effects of TBOEP, it was necessary to use not only environmentally relevant concentrations but also higher doses to fully understand the compound's impact. Liu et al., 2017, reported a 96-hour LC₅₀ of 3489 µg/L, while Ma et al., 2016 found a predicted no-observed effect concentration of 2.4 µg/L. No previous studies were found on the 24-hour LC₅₀ with the same conditions as this project.

Based on this information, **the chosen concentrations for zebrafish embryo exposures were 8.23, 28.77, 100.6, 352, and 1230 µg/L.** This range spans from environmentally relevant concentrations to higher doses that may potentially cause significant effects on the organism.

In the laboratory, stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. During the exposure period, fresh experimental solutions were prepared every day by dilution of the stock solutions in fish water (final DMSO concentration: 0.2% (v/v)) (Martínez et al., 2020).

3.2 Ecotoxicology Assay with Zebrafish

3.2.1 Breeding and Dose-Response Assay

Zebrafish adults routinely remain separated by sex in different tanks at the IDAEA facility. To obtain eggs, adults were placed the night before in reproduction tanks with a 3:2 female-to-male sex ratio. Reproduction tanks were placed on a black background, and a mesh was placed in each tank to prevent adult zebrafish from accessing the eggs. During the next morning tanks were checked for the presence of eggs at the bottom of the tank. Adult fish were removed from reproduction tanks and returned to their respective maintenance tanks and fertilized eggs were collected by pouring each tank through a sieve with mesh, which corresponded to 0 days post-fertilization (dpf).

The sieve with the eggs was passed through 600 mL of fish water (1L Milli-Q water, 45 mL Instant Ocean salt, and 10 mL CaSO₄) for five minutes. Next, the eggs were transferred to another container containing 600 mL of fish water and 1.2 mL of 0.01% methylene blue solution, where they were left for five minutes while agitated. Finally, they were transferred back to 600 mL of fish water. The eggs were distributed into two glass containers containing embryo water (1L Milli-Q water and 1.25 mL Instant Ocean salt) and incubated at 27 ± 1°C. Embryos were kept in clean embryo water until the beginning of the exposures, which started at 4 dpf as it is shown in *Figure 11*.

On the 3rd dpf, plates were prepared. Larvae were placed in six-well plates, with 10 larvae per well in 3 mL of fish water, incubated at 27 ± 1°C. Fish with morphological abnormalities were discarded. The next day (4 dpf), before the start of the exposure swim bladder inflation was assessed. Then, fish water in each well of the plates was replaced with 3 mL of TBOEP-contaminated water at different concentrations (8.23, 28.77, 100.6, 352, and 1230 µg/L). A control group was also included in which embryo water contained 0.2% of DMSO (solvent).

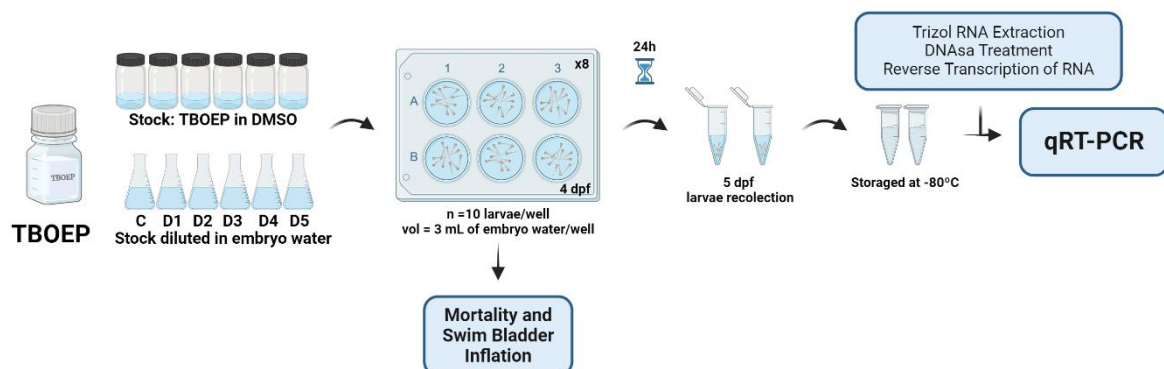


Figure 11 Methodology of zebrafish embryos exposure to TBOEP and process to obtain RT-RNA to apply to qRT-PCR

The fish were incubated at 27 (\pm 1°C). At the end of the exposures (5 dpf), swim bladder inflation and survival rates were recorded and quantified under a microscope. Embryos from each well were then transferred to Eppendorf tubes, where excess water was removed with a Pasteur pipette. The samples were frozen in dry ice and stored at -80°C until further analysis.

3.2.2 Sample Preparation for Transcriptomic Analysis

RNA Extraction

Samples were removed from the freezer and placed on dry ice. In each Eppendorf tube, 500 μ L of Trizol solution (Invitrogen Life Technologies) and 2 steel beads were added. The samples were then homogenized using a TissueLyser LT (Qiagen) at 40 Hz for 2 minutes and transferred to fresh tubes. Next, the samples were centrifuged at 12,000 rpm for 10 minutes at 4°C. The liquid phase was carefully transferred to the new Eppendorf and left to rest at room temperature for 5 minutes. Afterward, 100 μ L of chloroform (CHCl₃) was added, and the samples were manually agitated or vortexed. They were allowed to sit for 2-3 minutes at room temperature before being centrifuged again at 12,000 rpm for 15 minutes at 4°C. The supernatant was discarded using a pipette, and the remaining pellet was retained. To the pellet, 500 μ L of 75% ethanol was added without stirring or shaking. The samples were centrifuged for 5 minutes at 12,000 rpm at 4°C. Afterward, all ethanol was discarded, and the tubes were left to air-dry for 1 hour or until completely dry. Once dried, 20 μ L of diethylpyrocarbonated water was added to each tube, and incubated at 60 (\pm 1°C) for 10 minutes. Finally, the concentration of RNA was measured using a NanoDrop™ ND-8000 spectrophotometer (Fisher Scientific), and the RNA extraction were stored at -80°C for further use.

DNase – Desoxyribonuclease Treatment

From the NanoDrop™ reading, the volume required to add 5 μ g of total RNA to each Eppendorf was calculated. For each sample, 1 μ L of DNase was mixed with 5 μ L of buffer (100mM Tris pH 7.5; 25mM MgCl₂; 5mM CaCl). From this mixture, 4 μ L of the mix was added to each tube containing 5 μ g of RNA. Nuclease-free water was then added to adjust the final volume to 50 μ L. The reaction was then incubated for 30 minutes at 37 \pm (1°C). After incubation, the samples were placed in ice, and EDTA was added to a final concentration of 5nM. Finally, they were incubated at 75 (\pm 1°C) for 10 minutes, and the samples were kept in the ice for the next step of the procedure.

Inverse Transcription

This procedure was performed using the kit called Transcriptor cDNA Synthesis Kit by Roche Diagnostics. It was conducted in two steps:

a) RNA and primer denaturation:

Oligo(dT) primers were used to initiate the reverse transcription reactions. From the previous RNA preparation, where the concentration was 0,1µg/µL, 10 µL (containing 1 µg) was transferred into each new Eppendorf tube. To each tube, 1 µL of oligo(dT) primer and 2 µL of nuclease-free water were added. The tubes were incubated at 65 (± 1°C) for 10 minutes to allow the primers to anneal. Following the incubation, the tubes were immediately placed on ice to prevent further primer-RNA dissociation.

b) cDNA synthesis:

For each tube, there were added 4 µL of buffer (250mM Tris, 150 mM KCl, 40 mM MgCl₂), 2 µL of deoxynucleotide triphosphate (dNTP), 0.5 µL of RNA inhibitor, and 0.5 µL of reverse transcriptase enzyme. The reaction was incubated at 50 (± 1°C) for 1 hour to synthesize cDNA. Following this, the tubes were heated at 85 (± 1°C) for 5 minutes using a ThermoblockTM (Thermo Scientific) to inactivate the reverse transcriptase. The synthesized cDNA was stored at -80°C for further use.

3.2.3 Transcriptomic Analysis by Quantitative Real-Time PCR

Samples were analysed using a LightCycler 480 Real-Time PCR System (Roche Diagnostics). Each sample was run in duplicate in 96-well plates with a total volume of 20 µL per reaction. Each reaction mixture contained: 1 µg of RNA converted to cDNA (2 µL), 10 µL of SYBR Green Master (Roche Diagnostics), 300 nM (2 µL) of the appropriate primer set, and 6 µL of nuclease-free water. The reactions were performed using a LightCycler 480 Real-Time PCR System (Roche Diagnostics). The cycling parameters were as follows: an initial denaturation step of 10 minutes at 95°C, followed by 45 cycles of 10 seconds at 95°C and 30 seconds at 60°C. After amplification, a melting curve analysis was conducted to assess the specificity of the reaction. Two negative controls, non-template (sample replaced with sterile water) and RT-minus (without reverse transcription) were included on each plate to confirm the absence of primer dimers or genomic DNA contamination (Sanz et al., 2023).

Gene Selection and Primer Validation

Primer calibration was an essential step in quantitative RT-PCR to ensure that the primers used were specific, efficient, and produced reliable results. The calibration process involved optimizing primer concentration and confirming that the primers amplified the target sequence efficiently (aiming for optimal efficiency of 2) without producing non-specific products. The primers for this project were already designed in the Laboratory of Environmental Toxicology. My contribution involved conducting the melting curve analysis to verify the specific amplification of the chosen sequence. This step was performed after an acceptable number of cycles for different dilutions of the sample to ensure that the efficiency or standard curves were reliable. After optimization, the amplification efficiency of each primer pair was assessed by performing serial dilutions of cDNA under identical conditions. The resulting amplification curves showed an efficiency of $100 \pm 10\%$ and an r^2 value greater than 0.98.

The genes for this work were chosen from the literature review mentioned in 1.3, but also from the previous work done in the laboratory of environmental toxicology in IDAEA-CSIC after the analysis of the results from former studies of the flame retardant *TPHP* where some genes were altered and its comparison with TBOEP would help to understand if there is a common pathway of action between flame retardants.

To optimize resource management, an initial screening was conducted to identify the genes potentially affected by tris (2-butoxyethyl) phosphate. This screening involved analysing a mixture of all replicates, using just two technical replicates for each dose. Following the initial screening, three genes were selected for further investigation to study their effect in zebrafish embryos. Every gene corresponded to one PCR plate, every dose had at least 6 biological replicates and 2 technical replicates, which enabled the application of ANOVA, a statistical method used to compare the means of three or more doses to determine if at least one of the doses is statistically different from the others (Martínez et al., 2019). This focused approach allowed for a more in-depth analysis of the specific impact of TBOEP on gene expression and developmental outcomes in the zebrafish model.

Table 4 Genes selected to study the transcriptomic effect of TBOEP

Pathway	Gene symbol	Gene description	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
Housekeeping gen: Its expressions remain constant under xenobiotic affectations	<i>ppia 2</i>	peptidylprolyl isomerase Aa (cyclophilin A)	GGGTGGTAATGGAGCTGAGA	AATGGACTTGCCACCAGTTC
Detoxification: Drug-metabolizing enzyme mediator of phase I oxidative reactions	<i>ugt1a1</i>	UDP glucuronosyltransferase 1 family, polypeptide A1	CGTTTGATTCCCTTGTCGTCTTG	AACCACTGGAGTAGCGAGGC
Cellular response to xenobiotic stimulus: organic acid and xenobiotic metabolism, steroid hydroxylation, regulation of apoptotic process, antimicrobial humoral immune response, animal organ development, regulation of transcription by RNA polymerase II.	<i>cyp2k18</i>	cytochrome P450, family 2, subfamily K, polypeptide 18	CTGCATTCATCCAAGACCCAGT	TGGACACGCTCTGGGTTTG
	<i>cyp3a65</i>	cytochrome P450, family 3, subfamily A, polypeptide 65	GTCATGGTGCCGACCTACG	TCGGGTTTGAAGCTCTCCG
	<i>cyp26a1</i>	cytochrome P450, family 26, subfamily A, polypeptide 1	AACTACATCCCCTTCGGAGGA	TTGCAATGCTGCGTTAACTCA
Specific responses: response to estradiol, estrogen and steroid hormone, thiopurine drugs metabolism, response to drugs that modulate gluconeogenesis.	<i>pgr</i>	progesterone receptor	GGGCCACTCATGTCTCGTCTA	TCTCCACTCTGAAAATAT GTGGACTTT
Neurotoxic, related to myelination of nerves in the nervous system	<i>mbp</i>	myelin basic protein	TCTGTTGCTACATGCCTGCAG	GCATCACCTGACCATTGACAA
Alteration in hormone thyroid: Involved in G protein-coupled receptor and hormone-mediated	<i>tshβ</i>	thyroid stimulating hormone subunit beta	GTGTGCCCCCACTGACTACA	CCTGGAGAAACAGAAGCCC
Lipid binding activity: expressed in several structures, cardiovascular system, immature eye, muscle and nervous system	<i>fabp10a</i>	fatty acid binding protein 10a	TCGGCAAAGAGGCTGAAATC	TCCTCCATCCAGCTTGACG
	<i>fabp11a</i>	fatty acid binding protein 11a	GACGGCAAGGAGTCCACAAT	AGCCACCACATCACCCATCT
Involved in animal organ development face morphogenesis and myelination.	<i>aldh1a2</i>	aldehyde dehydrogenase 1 family, member A2	TGCTGCATCCTCATAGGCTG	CAGCTGGAATGGGTGTAGGC
Cellular response with phototransduction: located in eye, heart, integument, testis and visual system	<i>opn1mw2</i>	opsin 1 (<i>cone pigments</i>), medium-wave-sensitive, 2	CTCTTCGATAAATAGAGCGTGGG	AAGATGAAGCATCGGTGGAGA

3.2.4 Quantification of qRT-PCR results

Using the results from qRT-PCR, a relative quantification to assess the changes in gene expression across various TBOEP exposure levels was conducted. To reduce variability due to factors such as sampling, extraction, or enzymatic reactions, the data were normalized against a reference gene, *ppia2*, whose mRNA expression remained stable, whichever variance is present in its expression reflects this experimental variability. Normalization was achieved by comparing the expression levels using cycle threshold (Ct) values, which indicate the cycle at which fluorescence surpasses background noise. This was done following the qRT-PCR equation:

$$N = N_0(1 + E)^{Cp} \rightarrow N_0 = \frac{N}{(1 + E)^{Cp}}$$

Where N is the number of nucleic acid molecules, N_0 is the number of initial molecules, Cp is the number of cycles and E is the efficiency of the replication.

The number of normalized copies of the gen target from the reference gen is done through the following formula:

$$\frac{N_o^T}{N_o^R} = \frac{N_T / (1 + E_R)^{Cp^T}}{N_R / (1 + E_T)^{Cp^R}} = \frac{N_T}{N_R} \cdot \frac{(1 + E_R)^{Cp^R}}{(1 + E_T)^{Cp^T}}$$

For the calculations, I assume that the copy efficiencies are the same for all genes, either they are the optimum, $E = 100\%$, Therefore, the variance of the number of copies is the following:

$$E_R = E_T \rightarrow \frac{N_o^T}{N_o^R} = \frac{N_T}{N_R} 2^{Cp^R - Cp^T} \rightarrow \log_2 2^{Cp^R - Cp^T} = Cp^R - Cp^T = \Delta C_p$$

$$\Delta C_p = \log_2 \frac{N_o^T}{N_o^R}$$

Once all genes of treated and control samples are normalized for the reference gen, the number of times that the treated samples different from the control, named as *Fold Change*, is calculated.

$$FC = \frac{[Normalized\ copies\ N_o^T]_{treatment}}{[Normalized\ copies\ N_o^R]_{control}} = \frac{[2^{Cp^R - Cp^T}]_T}{[2^{Cp^R - Cp^T}]_C} = \frac{2^{\Delta C_p^T}}{2^{\Delta C_p^C}} = 2^{Cp^R - Cp^T}$$

$$\Delta \Delta C_p = \log_2$$

4. Results and Discussion

4.1 Survival and Swim Bladder Inflation Rate

After TBOEP exposure, survival rates were assessed to confirm that the doses were non-lethal. Additionally, and swim bladder inflation was quantified to determine whether the exposure had any adverse effects on this physiological function.

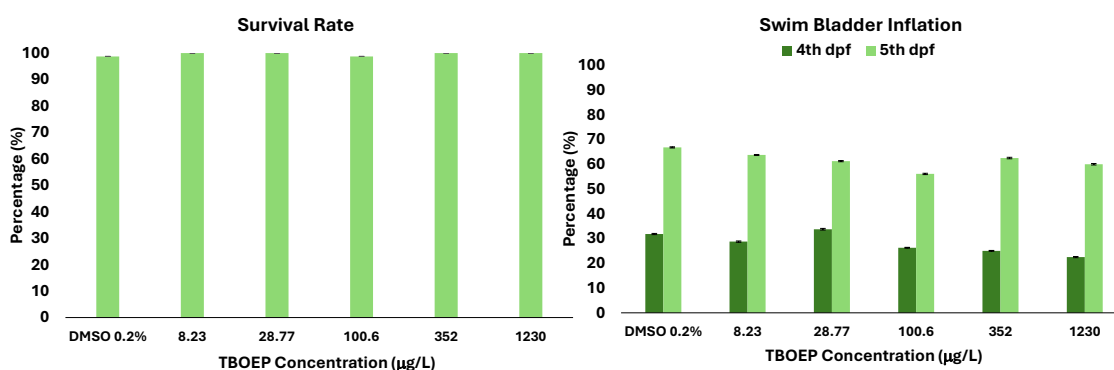


Figure 12. Survival rate for TBOEP exposure and Swim Bladder Inflation of zebrafish embryos at 4 dpf in comparison with 5 dpf for TBOEP

Our results show that no effects on survival and swim bladder inflation rates were detected in any of the tested concentrations (Figure 12). However, it is important to note that swim bladder inflation rates at 5 dpf in all experimental groups reached 60%, which it isn't common at this developmental stage of zebrafish larvae. This suggests that they may be underdeveloped for their age, which needs to be considered when taking conclusions of this experiment.

4.2 Effects of TBOEP at the Transcriptomic Level

First, a preliminary screening of the selected genes was carried out with a mixed sample containing all replicates from each experimental group, the results obtained are graphed in Figure 13. Since this analysis only resulted in a single data point per gene and experimental group, statistical analysis was not performed but trends in mRNA changes in the selected genes were observed.

In Figure 13 a tendency to downregulation is shown in genes *cyp3a65*, *cyp2k18*, *fabp10a*, *ugt1a1*, and *fabp11a*. For the case of *pgr*, the variance of technical replicates is too wide to confirm a tendency to upregulation. For the genes *mbp*, *cyp26a1*, *opn1mw2*, and *aldh1a2* no alteration is observed. In contrast, *tshβ* displayed a tendency to upregulation.

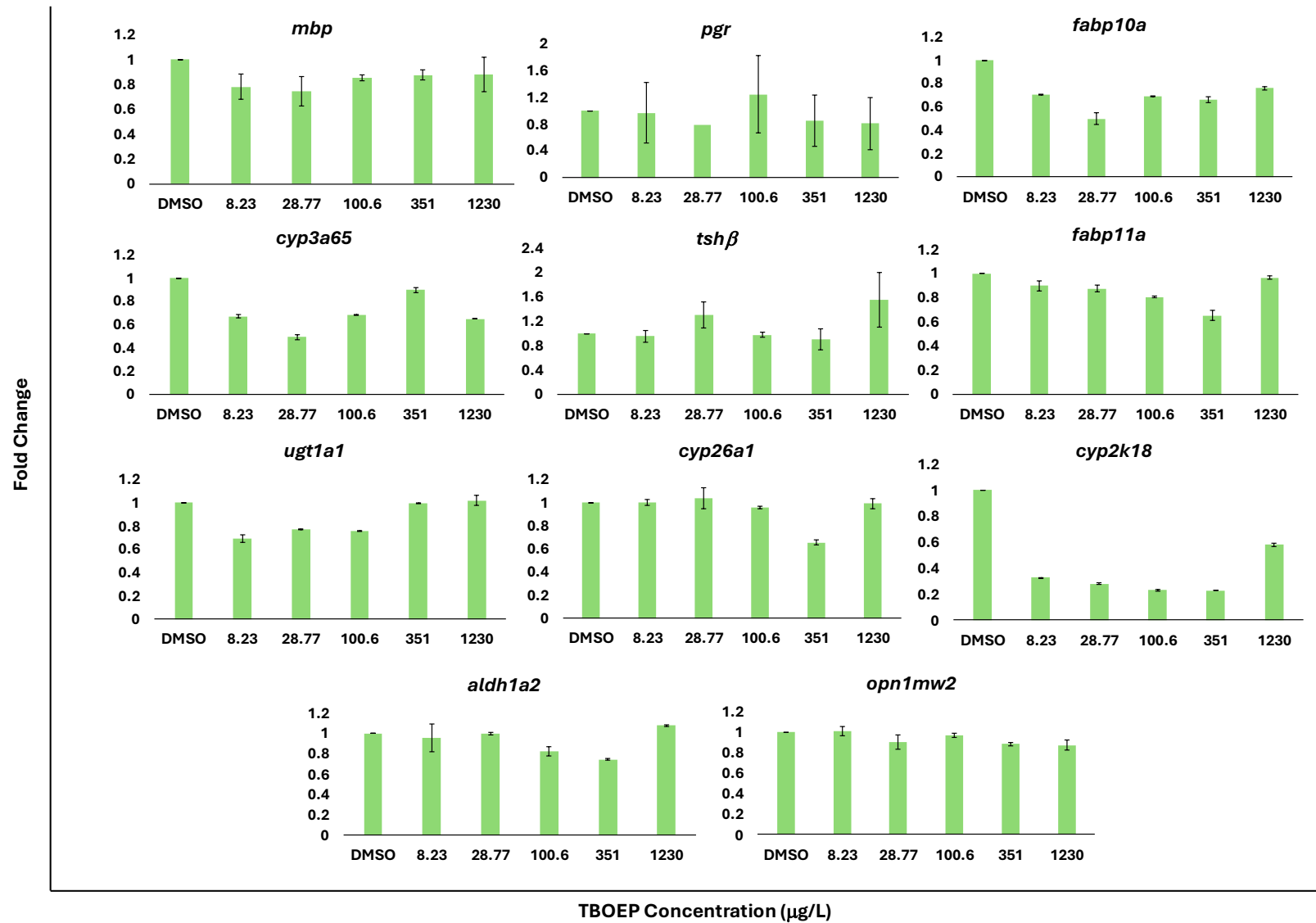


Figure 13 Fold change variation of several genes that were identified as biomarkers for genotoxicity. *mbp*, *pgr*, *fabp10a*, *cyp3a65*, *tshβ*, *fabp11a*, *ugt1a1*, *cyp26a1*, *cyp2k18*, *ald1a2* and *opn1mw2*, analysed as a pool mix of all replicates.

Second, a more in-depth analysis of the genes that showed the most prominent trends after exposure to TBOEP, which included 8 replicate samples from each experimental group (Figure 14). Our results showed that no significant differences were observed for *fabp10a* nor *cyp3a65*, while significant downregulation was observed in fish exposed to *cyp2k18*.

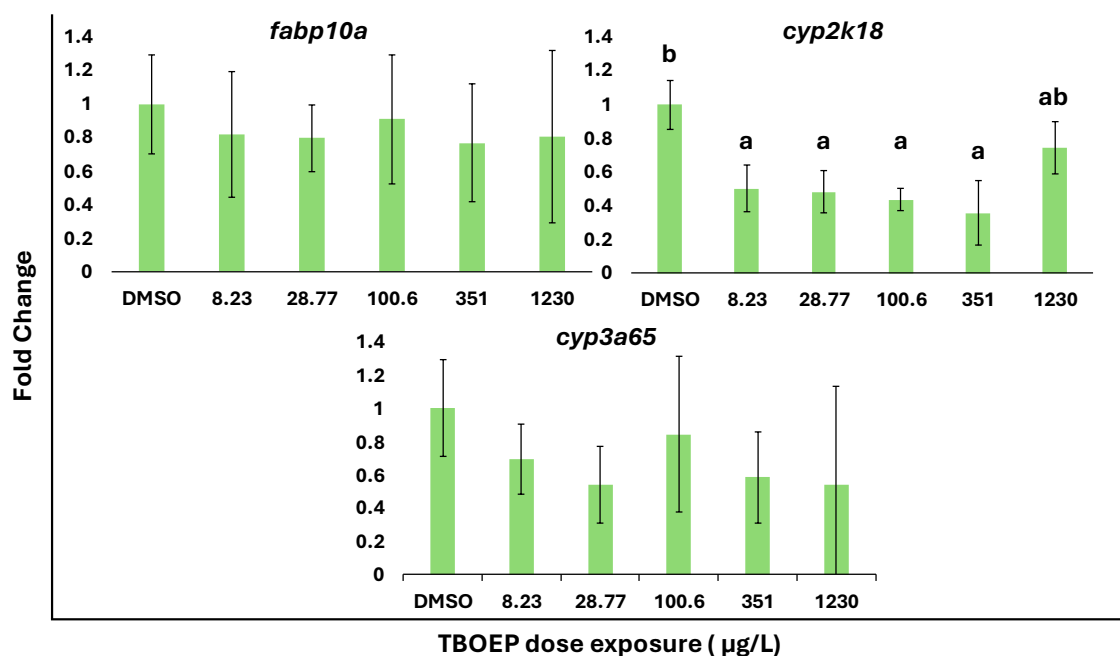


Figure 14 Fold change variation for three genes identified as biomarkers *fabp10a*, *cyp2k18* and *cyp3a65* expressed as the medium of the 8 replicates analysed separately.

Our results are inconsistent with those observed by Ma et al., 2015, who reported downregulation of *cyp3a65*, a gene associated with the pregnane X receptor, which is involved in the metabolism of various endogenous compounds and xenobiotics. They found fold changes of 0.45 and 0.39 at TBOEP doses of 80 µg/L and 200 µg/L, respectively. Additionally, Xu et al., 2023, observed a similar downregulation of *cyp3a65* in zebrafish embryos exposed to EHDPP, an aryl-OPFR, further supporting the notion that *cyp3a65* may respond to certain flame retardants. The discrepancies could be attributed to differences in exposure time and zebrafish larvae age. Their studies were conducted over 5 days, whereas our exposure lasted only 24 hours. Additionally, the results may vary due to differences in the methodology applied and the variance in biological replicates results.

Li et al., 2024 investigated the effects of TDMPP, an OPFR known to regulate estrogenic activity, on zebrafish embryos, and their study demonstrated that exposure to TDMPP resulted in the deregulation of *fabp10a*. During the differentiation period, estradiol (E2) played a critical role in liver development by reducing *fabp10a* expression. Consequently, a deregulation of this gene was expected for our sample. Another study,

Similarly, H. Cheng et al., 2022 found that nanoplastics increased their expression in larval livers, as the gene studied served as a biomarker of a hepatic inflammatory response to xenobiotic toxicants. Our results did not show statistically significant responses, and it may be attributed to the type of sample used, which consisted of whole larvae rather than liver tissue.

In contrast, the *cyp2k18* gene showed a more consistent response. Sanz et al., 2023's results identified *cyp2k18* as one of the three genes with the strongest toxic responses in organisms exposed to pollutants extracted from wastewater effluents, suggesting that this gene is highly sensitive to environmental contaminants. Additionally, Xu et al., 2023 observed that *cyp2k18* was upregulated after the exposure of EHDPP, indicating that this gene is involved in the detoxification process triggered by flame retardants. Both articles highlight *cyp2k18* as a reliable biomarker for identifying toxic effects in zebrafish embryos, which coincides with the results obtained in this project. Although the current study shows a tendency toward downregulation of *cyp2k18*, this result emphasizes the gene's role as a marker of toxicity and its potential involvement in the organism's detoxification pathways.

4.3 Environmental Implications in Aquatic Ecosystems

Tertiary treatment processes are essential in wastewater treatment plants as they are the only treatment that can significantly reduce the majority of OPFRs. While treatments like ozone (O_3) and UV/ H_2O_2 can break down certain alkyl OPFRs, they are ineffective against chlorinated OPFRs like TCEP (tris(2-chloroethyl) phosphate) and TCIPP (tris(1-chloro-2-propyl) phosphate). Additionally, these treatments often generate harmful by-products, including bromodichloromethane and chloroform (Cristale et al., 2016; Munné et al., 2023). Despite these limitations, tertiary treatments still offer a positive effect in reducing OPFR contamination, though further research is required to improve the efficiency of removal.

OPFRs are known to accumulate in sediments posing long-term risks to aquatic ecosystems (Cristale et al., 2013). When WWTP sludge containing OPFRs is applied to agricultural fields, runoff can introduce these contaminants into nearby water systems. The persistence and bioaccumulation of OPFRs raise significant concerns, particularly for aquatic organisms.

In this study, TBOEP (tris(2-butoxyethyl) phosphate) has been identified as a xenobiotic. In literature, TBOEP exposure in zebrafish embryos has been shown to affect various nuclear hormone receptor pathways and disrupt neuroendocrine functions (Ma et al., 2016b; Ju et al., 2023). Further research could focus on studying TBOEP's effects in single-cell liver samples, conducting further qPCR-RT analyses of affected genes, and extending exposure duration to better understand its toxicological profile (Van den Eede et al., 2015).

Another significant environmental concern is the interaction between OPFRs with other contaminants in systems. The presence of multiple pollutants can lead to additive or synergistic toxic effects that are not yet fully understood, further complicating risk assessments. Furthermore, despite the increasing use and recognized environmental effects of OPFRs, there remains a pressing lack of regulatory measures. While polybrominated diphenyl ethers (PBDEs) have been banned due to their toxicity, similar regulatory actions for OPFRs are still lacking. This regulatory gap underscores the urgent need for policymakers to reassess the environmental and health risks associated with OPFRs and to implement stronger controls to mitigate their impact.

5. Conclusions

Flame retardants are a highly diverse group of compounds with a great diversity of physical-chemical properties, having in common all of them bioaccumulated, but not all of them biomagnifies over the trophic chain. TBOEP is the flame-retardant compound with the highest concentration in the aquatic environment, and little research has been done on its effects at sublethal doses. For that reason, it was selected as the flame retardant to study in this thesis. From my literature review, TBOEP exposure in zebrafish embryos has been shown to affect various nuclear hormone receptor pathways, and developmental processes, produce oxidative stress, alter DNA methylation, and induce neurotoxicity.

Regarding the experimental results, we demonstrated that the doses tested were sublethal. *cyp2k18* gene expression showed a tendency to be downregulated after exposure to TBOEP, but for other genes, no clear trends were observed. Therefore, we conclude that TBOEP activates detoxification pathways and xenobiotic metabolism, and it is necessary to continue studying and delving into what type of effect it generates.

Emerging contaminants at nano-level concentrations are an especially important focus of study because it is not known exactly if the environmentally relevant concentrations are already affecting organisms. The growing body of research on TBOEP and other OPFRs highlights the need for a more comprehensive understanding of their risks, especially in regions like Catalonia where water reuse is essential. Effective wastewater treatment technologies must be developed and implemented to address the limitations of current systems. Moreover, regulatory frameworks should be updated to reflect the emerging threat of OPFRs and other CECs. Improved monitoring and risk assessments are necessary to safeguard both aquatic ecosystems and human health from the long-term effects of these contaminants.

For future lines of work, I identify a) study the effects of other types of flame retardants, b) study the effect of the combination of flame retardants on zebrafish, and c) investigate methods to increase the efficiency of removing nano-level concentrations of contaminants from wastewater.

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7. Supplementary Data

7.1 Literature Review of Genes Altered and Their Respective Fold Change

In the table below, five articles about zebrafish exposed to TBOEP were analysed to further understand which genes were affected by the compound.

Table S1 Upregulated genes obtained from the literature review; 1- Q. Xu et al., 2017, 2-Ma et al., 2016b, 3-Ma et al., 2015, 4- Liu et al., 2017.

GENE NAME	GEN ID	BIOLOGICAL FUNCTION	TYPE OF CELL	DEVELOPMENT	EXPOSITION TIME	DOSIS	CONCENTRATION WITH SIGNIFICANT FOLD CHANGE	FOLD CHANGE	REFERENCE
<i>17βhsd</i>	AY306005	regulation of steroid metabolism	gonad	adult female	21 days	0, 5, 50 and 500 ug/L	50, 500 ug/L	>2	1
<i>3βhsd</i>	AY279108	regulation of steroid metabolism	gonad	adult female	21 days	0, 5, 50 and 500 ug/L	50, 500 ug/L	1-1.5, >2	1
<i>cyp17</i>	AY281362	cytochrome P450	gonad	adult female	21 days	0, 5, 50 and 500 ug/L	50, 500 ug/L	>2	1
<i>er α</i>	NM 152959	estrogenic receptor	liver	adult female	21 days	0, 5, 50 and 500 ug/L	500 ug/L	>2	1
<i>star</i>	NM 131663	steroidogenic acute regulatory protein	gonad	adult female	21 days	0, 5, 50 and 500 ug/L	500 ug/L	>2	1
<i>vtg1</i>	AF406784	vitellogenin	liver	adult female	21 days	0, 5, 50 and 500 ug/L	500 ug/L	>2	1
<i>vtg3</i>	AF254638	vitellogenin	liver	adult female	21 days	0, 5, 50 and 500 ug/L	500 ug/L	>2	1
<i>17βhsd</i>	AY306005	regulation of steroid metabolism	gonad	adult male	21 days	0, 5, 50 and 500 ug/L	50, 500 ug/L	1-1.5, >2	1
<i>cyp19a</i>	AF226620	cytochrome P450 19	gonad	adult male	21 days	0, 5, 50 and 500 ug/L	500 ug/L	>2	1
<i>hmgrb</i>	NM 001014292	hydroxymethylglutaryl CoA reductase	gonad	adult male	21 days	0, 5, 50 and 500 ug/L	500 ug/L	>2	1
<i>17βhsd</i>	AY306005	regulation of steroid metabolism	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	2, 20 ug/L	2.7	2
<i>ar</i>	NM_001083123	androgen receptor	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	2 ug/L	2	2
<i>ccnd1</i>	NM_131025	strogen receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.02 uM	2.3	3
<i>crhr2</i>	XM_681362	corticotrophin-releasing hormone receptor 2	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	2 ug/L	2.18	2
<i>deio1</i>	BC076008	activate and inactivate thyroid hormones, crucial for maintaining the T4 and T3 ratio	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	1000 and 2000 ug/L	2.7	4
<i>deio2</i>	NM_212789	crucial for maintaining the T4 and T3 ratio	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	1000 and 2000 ug/L	2.3	4
<i>dut</i>	NM_001006005	peroxisome proliferator-activated receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.02, 0.1 uM	2.50, 1.81	3
<i>er1</i>	NM_152959	Estrogen receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.1, 0.5 uM	1.83, 2.4	3
<i>er2a</i>	NM_180966	Estrogen receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.1, 0.5 uM	1.96, 2.18	3
<i>gnrhr4</i>	NM_OO1098193	gonadotropin-releasing hormone receptors	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	2 ug/L	1.9	2
<i>gr</i>	EF567112	glucocorticoid receptor	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	2 ug/L	2.14	2
<i>pgr</i>	NM_001166335	Estrogen receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.5 uM	2.48	3
<i>pou1f1</i>	NM_212851	pregnane x receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.5 uM	1.99	3
<i>rela</i>	AY163839	glucocorticoid receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.5 uM	2.07	3
<i>tg</i>	XM_001335283	thyroglobulin, gene involved in thyroid synthesis	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	2 ug/L	2	2
<i>tg</i>	XM_001335283	thyroglobulin, thyroid synthesis pathway	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	1000 and 2000 ug/L	2.5	4
<i>tsnr</i>	NM_001145763	thyroglobulin, thyroid synthesis pathway	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	2, 20 ug/L	2.24, 1.97	2
<i>ugt1a1</i>	NM_001037428	genes related to thyroid hormone metabolism	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.02 uM	2.56	3
<i>vtg5</i>	NM_001025189	estrogen receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.5 uM	2.03	3

Table S2 Downregulated genes obtained from literature review; 1- Q. Xu et al., 2017, 2-Ma et al., 2016b, 3-Ma et al., 2015, 4- Liu et al., 2017.

GENE NAME	GEN ID	BIOLOGICAL FUNCTION	TYPE OF CELL	DEVELOPMENT	EXPOSITION TIME	DOSIS	CONCENTRATION WITH SIGNIFICANT FOLD CHANGE	FOLD CHANGE	REFERENCE
<i>ar</i>	NM_001083123	androgen receptor	brain	adult female	21 days	0, 5, 50 and 500 ug/L	500 ug/L	0.5-1	1
<i>cyp19a</i>	AF226620	cytochrome P450 19	gonad	adult female	21 days	0, 5, 50 and 500 ug/L	50, 500 ug/L	<0.5	1
<i>fsh β</i>	NM_205624	follicle stimulating hormone	brain	adult female	21 days	0, 5, 50 and 500 ug/L	500 ug/L	<0.5	1
<i>gnrh3</i>	NM_182887	gonadotropin-releasing hormone	brain	adult female	21 days	0, 5, 50 and 500 ug/L	50, 500 ug/L	<0.5	1
<i>gnrh3</i>	NM_001177450	gonadotropin-releasing hormone receptor	brain	adult female	21 days	0, 5, 50 and 500 ug/L	500 ug/L	<0.5	1
<i>hmgra</i>	BC155135	hydroxymethylglutaryl CoA reductase	gonad	adult female	21 days	0, 5, 50 and 500 ug/L	500 ug/L	<0.5	1
<i>lh β</i>	NM_205622	lutinizing hormone	brain	adult female	21 days	0, 5, 50 and 500 ug/L	500 ug/L	<0.5	1
<i>hmgra</i>	BC155135	hydroxymethylglutaryl CoA reductase	gonad	adult male	21 days	0, 5, 50 and 500 ug/L	5, 50, 500 ug/L	<0.5	1
<i>lh β</i>	NM_205622	lutinizing hormone	brain	adult male	21 days	0, 5, 50 and 500 ug/L	50, 500 ug/L	<0.5	1
<i>11βhsd</i>	AY578180	mineralocorticoid receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.02, 0.1 uM, 0.5 uM	0.64, 0.42, 0.31	2
<i>adrb2b</i>	NM_001089471	mineralocorticoid receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.1 uM, 0.5 uM	0.24, 0.33	3
<i>crh</i>	NM_001007379	In teleosts, TSH secretion is thought to be stimulate by it	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	10, 200, 1000, 2000 ug/L	0.5	2
<i>cyp11a</i>	NM_152953	cholesterol side-chain cleavage enzyme	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	2, 200 ug/L	0.5	2
<i>cyp11a</i>	NM_152953	cholesterol side-chain cleavage enzyme	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	2, 200 ug/L	0.5	2
<i>cyp17</i>	AY281362	estrogen metabolic enzyme	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	20 ug/L	0.5	2
<i>cyp24a1</i>	NM_001089458	pregnane x receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.1 0.5 uM	0.52, 0.46	3
<i>cyp3a65</i>	AY452279	pregnane x receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.1 uM, 0.5 uM	0.65, 0.45, 0.39	3
<i>fshβ</i>	AY424303	follicle-stimulating hormoneβ	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	20 ug/L	0.4	3
<i>ghra</i>	NM_001083578.1	gen involved in GH/IGF	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	2000 ug/L	<0.5	4
<i>ghrb</i>	NM_001111081.1	gen involved in GH/IGF	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	2000 ug/L	<0.5	2
<i>hdac3</i>	NM_200990	thyroid hormone receptor alpha	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.1, 0.5 uM	0.57, 0.39	2
<i>igf1</i>	NM_131825.2	ligand highly expressed in embryogenesis	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	2000 ug/L	<0.5	4
<i>igf1ra</i>	NM_152968.1	ligand highly expressed in embryogenesis	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	1000 and 2000 ug/L	0.5	4
<i>igf2b</i>	NM_001001815	ligand highly expressed in embryogenesis	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	2000 ug/L	0.4	4
<i>igf3</i>	NM_001115050.1	ligand highly expressed in embryogenesis	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	10, 200, 1000, 2000 ug/L	<0.3	4
<i>igfbp3</i>	NM_205751.2	bind circulating IGFs and modulate their actions	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	200, 1000, 2000 ug/L	0.5	4
<i>igfbp6a</i>	NM_001161401.1	bind circulating IGFs and modulate their actions	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	1000 and 2000 ug/L	0.5	4
<i>igfbp6b</i>	NM_001161402.2	bind circulating IGFs and modulate their actions	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	2000 ug/L	0.6	2
<i>il6</i>	JN698962	peroxisome proliferator-activated receptor alpha	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.1, 0.5 uM	0.78, 0.45	2
<i>mr</i>	EF567113	mineralocorticoid receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.1, 0.5 uM	0.68, 0.42	2
<i>ncoa1</i>	XM_686652	Estrogen receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.1 uM	0.37037037	2
<i>ncoa2</i>	NM_131777	Estrogen receptor	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.02	0.649350649	2
<i>nis</i>	NM_001089391	ansports sodium and iodide in synthesis of thyroid hormon	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	200 ug/L	0.3	2
<i>nis</i>	NM_001089391	ansports sodium and iodide in synthesis of thyroid hormon	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	10, 200, 1000, 2000 ug/L	0.8, <0.5	4
<i>pomc</i>	AY158003	pro-opiomelanocortin	fullbody	larvae	3 hpf to 120 hpf	2, 20, 200 ug/L	20, 200 ug/L	0.5	3
<i>pparg</i>	NM_131467	peroxisome proliferator-activated receptor alpha	fullbody	larvae	3.5hpf to 120 hpf	0, 0.02, 0.1 and 0.5 uM	0.02, 0.1, 0.5 uM	0.64, 0.69, 0.53	3
<i>tr β</i>	NM_001014292	control the development and physiology of fish primarily	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	200, 1000, 2000 ug/L	<0.6	2
<i>thrl1</i>	NM_001114688	thyrotropin-releasing hormone receptor	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	1000 and 2000 ug/L	0.5	2
<i>tsh β</i>	AY135147	which regulates the synthesis of THs	fullbody	larvae	2 hpf to 144 hpf	0, 10, 200, 1000, 2000 ug/L	10, 200, 1000, 2000 ug/L	0.5	2

7.3 RNA Concentration After Extraction and Purity Index

RNA concentrations from the sample from 8 replicates are between 337 and 1390 ug/L. Samples 1 D2, 1 D3, 1 D5, 2 D2, and 8 D4 had to be displaced from the continuity of the experiment because of low concentration, and further analysis would be without results.

About the purity index, the 260/280, which range from 1.68 to 2.00, are acceptable for all samples. The ratio 260/230 is adequate for all sample which has good RNA concentration because in some cases, it corresponds to 0,14, which is out of the acceptable range from 1.00 to 2.00 to use it for RT-PCR if it would be used for RNA-seq, the range should be reduced to 1.80 to 2.00.

Table S3 RNA concentration from extractions from zebrafish embryos for 8 replicates. RNA concentration is indicated in ng/μl, purity index 260/280 and 260/230 go without units.

Sample ID	RNA-40 ng/ul	260/280	260/230	Sample ID2	RNA-40 ng/ul3	260/280 4	260/230 2
1 C	930,3	1,90	1,93	5 C	620,1	1,96	1,96
1 D1	825,3	1,87	1,89	5 D1	787,5	1,90	1,96
1 D2	39,36	1,83	0,14	5 D2	513,6	1,89	1,84
1 D3	4,126	1,68	0,04	5 D3	625,5	1,82	2,14
1 D4	725,8	1,80	1,63	5 D4	541,1	1,87	2,13
1 D5	3,641	1,84	0,04	5 D5	757,6	1,89	2,12
2 C	747,9	1,85	2,17	6 C	771,4	1,98	1,38
2 D1	648,3	1,92	1,02	6 D1	688,4	2,00	1,78
2 D2	6,277	1,88	0,03	6 D2	648,7	1,85	2,28
2 D3	619,5	1,79	1,56	6 D3	527,5	1,88	1,95
2 D4	777,0	1,81	2,11	6 D4	531,4	1,85	2,15
2 D5	716,1	1,90	1,42	6 D5	337,4	1,90	1,77
3 C	895,2	1,95	2,26	7 C	917,1	1,87	2,08
3 D1	608,3	1,83	2,02	7 D1	449,6	1,90	1,69
3 D2	650,2	1,87	2,02	7 D2	630,6	1,85	2,00
3 D3	1019,00	1,91	2,02	7 D3	747,9	1,87	1,97
3 D4	847,3	1,96	1,61	7 D4	857,3	1,96	1,71
3 D5	730,4	1,98	2,05	7 D5	897,1	1,93	1,88
4 C	1390,00	1,98	1,78	8 C	1029,00	1,87	2,17
4 D1	851,8	1,93	2,15	8 D1	927,2	1,86	2,34
4 D2	756,2	1,95	1,64	8 D2	1077,00	1,91	0,55
4 D3	1123,00	1,93	2,17	8 D3	951,3	1,86	2,40
4 D4	914,9	1,94	1,95	8 D4	73,30	1,97	0,53
4 D5	918,0	1,96	2,32	8 D5	760,2	1,88	2,31

7.4 Fugacity Model's Methodology

The fugacity model is a useful tool for assessing the behaviour and fate of organic pollutants across different environmental compartments, such as during wastewater treatment, in surface water bodies. This model is based on the concept of fugacity, which describes the tendency of a chemical to “escape” from one phase to another and is used as an equilibrium criterion. Fugacity is denoted as f and measured in Pa.

When a pollutant moves between two compartments, the model assumes that equilibrium is reached when the fugacity is equal in both phases, the pressure of the chemical in both phases should balance out.

The relationship between fugacity and concentration is given by the equation:

$$C = Z \cdot f$$

Where:

- C is the concentration of the pollutant in a given phase ($mol\ m^{-3}$),
- Z ($mol\ m^{-3}\ Pa^{-1}$) is the fugacity capacity, which is specific to each chemical and depends on the phase and environmental conditions. A compartment with a higher fugacity capacity can hold greater concentration for a given pollutant.

From a known concentration of a compound in one phase, the fugacity can be deduced or vice versa. This helps in modelling the transport and transformation of chemicals across separate phases, such as water, biomass, or air.

The movement of chemicals between different compartments occurs via transport and transformation processes. These processes include degradation and surface volatilization, with their rates being described by D values.

$$D = K \cdot V \cdot Z \ (mol\ h^{-1}\ Pa^{-1})$$

Where:

- D is the rate of transport or transformation
- K is a rate constant or removal mechanism coefficient
- V is the volume of the compartment
- Z is the fugacity capacity of the phase

To calculate D values, it is essential to know the Z values, information related to the compound (such as removal mechanisms and rate constants), and specific data from the

wastewater treatment plant (like the volume of sewage tank and mass flows) (Y. Wang et al., 2020).

In this project, three key phases were analysed:

1. Water
2. Fish
3. Fish's diet

These compartments were chosen to model the accumulation of pollutants and evaluate the fugacity across these environmental media. Several parameters were calculated to derive the fugacity in each phase.

1. Bioaccumulation Factor (BCF)

The accumulation of pollutants in fish's organisms occurs through different pathways, such as uptake from water or diet. The BCF is used to describe this process:

$$BCF = \frac{Z_{fish}}{Z_{pathway}}$$

Z_{fish} is the fugacity capacity of the fish.

$Z_{pathway}$ is the fugacity capacity of the pathway (e.g. water or diet)

2. Fugacity capacity (Z)
 - a) Air:

The concentration of a chemical in air can be described as:

$$C_{i,air} = Z_{air} \cdot f$$

Using the ideal gas law, we can express the concentration in air:

$$p_{i,air} \cdot V_i = n_i \cdot R \cdot T \rightarrow C_{i,air} = \frac{p_i}{R \cdot T}$$

Assuming $f = p_i$, the fugacity capacity in air becomes:

$$Z_{air} = \frac{1}{R \cdot T}$$

b) Water:

For water, the concentration is related to the Henry's Law Constant (K_H):

$$C_{i,w} = Z_w \cdot f$$
$$K_H = \frac{p_i}{c_{i,w}} = \frac{f}{c_{i,w}}$$

Thus, the fugacity capacity in water is:

$$Z_w = \frac{1}{K_H}$$

c) Biota:

For biota, the fugacity capacity is related to the lipid content and octanol-water partition coefficient (K_{ow}):

$$C_{i,biota} = Z_{bio} \cdot f$$

The partitioning between biota and water is expressed as:

$$K_{i,biota} = \frac{C_{i,biota}}{C_{i,w}} = f_{lip,i} \cdot K_{ow}$$

Thus, the fugacity capacity in biota becomes:

$$Z_{bio} = f_{lip} \cdot K_{ow} \cdot \frac{1}{K_H} \cdot \rho_{bio}$$

Where:

- f_{lip} is the fraction of lipids in the organism
- ρ_{bio} is the density of the biological tissue

In order to quantify the concentration at equilibrium between states, the fugacity of each has to be quantified in a relative value, in proportion with other states. Therefore, the fugacity of a organism:

$$f_{bio} = \frac{D_w \cdot f_w + D_d \cdot f_d}{D_R + D_E + D_B + D_C}$$

