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Transformation of Tributyltin in Zebrafish Eleutheroembryos (*Danio rerio*)

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Abstract Organotin compounds are highly versatile group of organometallic chemicals used in industrial and agricultural applications. Their endocrine-disrupting effects are well known and their extensive uses as biocide materials, e.g., in antifouling paints, for many years have led to serious environmental problems. So far, attention has mainly been given to tributyltin pollution in water, sediments, and marine organisms because of its highly toxic effects and high accumulation levels at very low concentrations. In this study, we will focus on the conversion of tributyltin after it is absorbed by zebrafish eleutheroembryos, presented here as an alternative model to adult fish for describing bioconcentration. A simplified analytical extraction procedure based on the use of an assisted ultrasonic probe and derivatization by ethylation, followed by gas chromatography with a flame photometric detector (GC-FPD) is proposed. This classical methodology for organotin determination has been validated by inductively coupled plasma mass spectrometry (ICP-MS) and Zeeman graphite furnace atomic absorption spectrometry (ZGF-AAS) in terms of total tin content. The speciation analysis results show that zebrafish eleutheroembryos absorb high amounts of tributyltin and convert it into monobutyltin and likely in inorganic tin.

Keywords Zebrafish eleutheroembryos · Organotin compounds · Detoxification

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Introduction

The toxicological pattern of organotin compounds (OTCs) is very complex. Their biological effects depend on both the nature and the number of the organic groups bound to tin [1]. The high toxicity of these compounds towards nontarget organisms, particularly in the marine ecosystem, can cause ecological damage and affect human health through the food chain [2, 3].

Thus, strict legislative restrictions have been introduced in 25 Western countries in order to help reduce the accumulation of these compounds in the affected areas [4]. In 2001, the International Maritime Organization (IMO) sponsored the International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention). The aim of this convention is to protect the marine environment and human health from the adverse effects of anti-fouling systems on ships by phasing out the use of harmful OTCs, such as biocides in anti-fouling paints, and establishing mechanisms to prevent the potential future use of other harmful substances [5]. The convention became effective in September 2008, banning the use of tributyltin (TBT) in anti-fouling paints on ships [6]. Furthermore, the commercialization and use of OTCs within the EU was prohibited as of January 1, 2003, by another Commission Directive [7]. Consequently, TBT, the most widely used organotin, has been included in the European Union List of Priority Substances and Certain Other

Pollutants, in the field of water policy for which environmental quality standards were set in 2008. The last update of the list was done on September 21, 2012 (http://ec.europa.eu/environment/water/water-framework/priority_substances.htm). However, it seems that the implementation of the AFS Convention in developing countries [8] appears to be difficult due to lack of analytical resources. Detection level of organotin compounds such as methyl butyltin, dibutyltin, tributyltin, and triphenyltin is low in developed countries because organotin health risks are under control, unlike the developing countries where their use is not strictly regulated [9]. Furthermore, the treatment and disposal of shipyard water contaminated by organotins is a serious concern in those areas. The permanent presence of these contaminants in estuarine sediments and marine organisms is easily explained if the previous factors are considered, as well as the low degradation rate of TBT, particularly in anoxic and/or cold environments [10, 11].

The fate of TBT in aquatic ecosystems and the ecotoxicological effects directly depend on its persistence and the occurrence of biotic and abiotic degradation mechanisms [12]. TBT degradation involves the sequential removal of butyl groups, leading to the formation of di- and monobutyltin (DBT and MBT). These dealkylation processes generally result in a reduction of toxicity. Many reports have already evaluated the metabolism in adult fish, demonstrating that TBT is rapidly degraded into the metabolites DBT and MBT that are then transferred to bile and eliminated [13, 14]. However, another aspect when controlling the fate and persistence of TBT in aquatic systems is directly linked to the partitioning processes between water, sediments, and biota through biotransformation. Different studies carried out in marine organisms have reported significant bioconcentration of butyltin compounds in benthic macro-organisms, feeding on sediment particles from estuarine and coastal samples, but low degradation by dealkylation processes [15, 16].

On the other hand, early life stages seem to be more sensitive to TBT pollution than adult individuals. Larval bivalve mollusks and juvenile crustaceans appear to be much more sensitive than adults during acute exposures. The 96-h LC_{50} for the larval Pacific oyster, *Crassostrea gigas*, was 1557 μg L^{-1}, whereas the value for adults was 282 μg L^{-1} [17]. Another test with the fathead minnow, *Pimephales promelas*, caused the death of the whole population after 32 days of exposure to 2 μg L^{-1} TBT [18].

A previous investigation made by our group [19] was based on the need to study the toxicity, persistence, and bioaccumulation of persistence, bioaccumulation, and toxic substances (PBTs), regulated by the European REACH legislation. Zebrafish eleutheroembryos (*Danio rerio*) were chosen as the biological model because they have a series of advantageous features over other vertebrate models, mainly rapid embryonic development, and the fact that they can be easily maintained. In such study, we analyzed eleutheroembryo bioconcentration capability when exposed to 0.2 and 2 μg L^{-1} of TBT, corresponding to 0.1 and 1 % of TBT LC_{50}, as described in the literature [20, 21]. The bioconcentration was assessed by evaluating bioconcentration factors (BCFs) which account the substance concentration ratio measured in the exposure medium and eleutheroembryos tissues. The results revealed that total tin concentration inside zebrafish eleutheroembryos increased with the time of exposure and decreased during the depuration phase.

The aim of this study is to complement the TBT accumulation data observed in zebrafish eleutheroembryos by measuring the organotin species present in the latest stages of the absorption phase and during depuration. The main analytical challenge was the very small weight of this type of biological samples, around 20 mg for 15–20 eleutheroembryos and their high fat content. For the extraction technique, a minimum amount of extractant was used to avoid high analyte dilution. The speciation study was done by gas chromatography-flame photometric detector (GC-FPD) after OTCs ethylation with sodium tetraethylborate ($NaEt_4B$). Inductively coupled plasma mass spectrometry (ICP-MS) and Zeeman graphite furnace atomic absorption spectrometry (ZGFAAS) were employed to validate total tin content by comparing the total amount of species after the chromatographic procedure with the total tin measured using the described techniques.

Experimental

Instrumentation

Organotin speciation was performed in a gas chromatograph HP-5890-Series II, equipped with a ZP-5 column (30 m× 0.25 mm). The column temperature was programmed to 1 min at 70 °C, followed to an increase to 250 °C (at 25 °C min^{-1}), and a final temperature of 290 °C held for 2 min. The FPD detector was provided with a 610-nm cut-off filter.

Total tin concentration was monitored by a PerkinElmer 4100 ZL atomic absorption spectrometer with a longitudinal Zeeman background correction, equipped with a transversally heated graphite furnace tube atomizer (THGA) with L'vov platform. Tin concentration was calculated from the integrated absorbance of the atomic absorption signal. A volume of 20 μL was injected manually. The furnace operation was controlled using the PerkinElmer AA Winlab software, Version 4.1 SSP1. A PerkinElmer hollow cathode lamp (HCL)

with wavelength 286.3 nm and instrument slit width 0.7 nm was used. Alternatively, an ICP-MS HP-7700 Plus (Agilent Technologies, Analytical System, Tokyo, Japan), equipped with a Babington nebulizer, Fasssel torch, and double-pass Scott-type spray chamber cooled by a Peltier system was used. Single ion monitoring at m/z 118 and 120 was used for obtaining the data.

A Vibracell VCX130 ultrasonic processor (Connecticut, USA), equipped with a 2-mm diameter titanium microtip and fitted with a high-frequency generator of 130 W at 20 kHz was used to leach OTCs from eleutheroembryos. Centrifugation was carried out in a centrifuge model type FVL-2400N (Combi-Spin, Boeco, Germany).

Reagents

Analytical grade chemicals were used for all the experiments in this work. The standards for TBTCl (>97 %), $DBTCl_2$ (>97 %), $MBTCl_3$ (>95 %), and TPrTCl (97 %) were obtained from Sigma-Aldrich Quimica S.A. (Madrid, Spain). The organotin stock solutions containing 1000 mg L^{-1} as tin were prepared in pure methanol and stored at 4 °C in the dark. Glacial acetic acid was purchased from Panreac Química S.A. (Madrid, Spain); sodium acetate, methanol, and hexane were supplied by Scharlab S.L. (Barcelona, Spain). All solutions and samples were prepared using ultrapure water obtained from a Millipore (Bedford, MA, USA) ZMFQ 23004 Milli-Q water system. Sodium tetraethylborate ($NaBEt_4$) was from Sigma-Aldrich Quimica S.A. (Madrid, Spain); the corresponding 1 % aqueous solutions (in Milli-Q water) for derivatization were prepared daily under nitrogen atmosphere. The candidate RM oyster tissue T-37 (IRMM, Belgium) was employed for both optimization and validation of the analytical method.

Procedure for Zebrafish Eleutheroembryos Exposure

The Marine and Food Technological Centre of the Basque Country (Azti-Bilbao, Spain) supplied zebrafish eleutheroembryos. The exposure solution had a composition similar to that of fresh river water: 16 mL of the concentrated solution (containing 2.9 g of $CaCl_2$, 17.2 g of NaCl, 0.76 g of KCl, and 4.9 g of $MgSO_4$) were diluted to 1 L with distilled water. Following the OECD guidelines, the conditions of the exposure solution were 26±2 °C, dissolved oxygen ≥60 %, and pH 6–8.5.

To obtain the samples, embryos were allowed to develop for 72 h post-fecundation (hpf), at which time the embryos hatched. For the absorption phase, the appropriate number of eleutheroembryos was placed into a tank containing 2 μg L^{-1} of TBT and left for 45 to 48 h. Next, the eleutheroembryos were moved to a tank with clean exposure solution for the depuration phase analysis. Approximately, groups of 15–25 eleutheroembryos were removed from the tank after 45 and 48 h (absorption phase), and 60 and 72 h (depuration phase).

Extraction Procedure for OTC Speciation

OTC extraction from eleutheroembryos was done by adding 200 μL of acetic acid and 200 μL of methanol to a vial containing approximately 20 mg of the sample, accounted by estimating the individual eleutheroembryos weight and multiplying it by the individual number contained in each vial. The ultrasonic probe was immersed into the vial for 60 s at 50 % of its amplitude, and the resulting suspension was centrifuged at 4000 rpm for 10 min. Approximately 200 μL of the supernatant was taken and the pH adjusted to 4.8 with acetic acid/acetate buffer. Next, 500 μL of 1 % $NaBEt_4$ was added for derivatization lasting 5 min and the derivatized analytes were extracted into 200 μL of hexane. The solution was stirred for 15 min and an aliquot of 1 μL injected into the GC-FPD for chromatographic analysis.

Development and Validation of the Quantification Method

OTCs speciation was carried out by GC-FPD as described above. Standard addition calibration curves for each compound were established by spiking increasing concentration of TBT, DBT, and MBT on nonexposed eleutheroembryos. This was the best approach due to the high lipidic content of the samples. Furthermore, the likely errors associated to the derivatization process and the GC-FPD measurements were corrected by spiking 100 μL of 50 μg L^{-1} of tripropyltin as the internal standard after the extraction procedure. The linearity range was comprised between DL and 50 μg L^{-1}. The detection limit was established at 2 μg L^{-1} for the three studied compounds (MBT, DBT, and TBT), being enough for the samples analyzed whose contained a high amount of OTCs due to the high accumulation observed [19]. Validation of the speciation method was performed by comparing the total tin content as the total amount of species of the chromatographic method with the amount of tin measured in the same samples by ZGF-AAS and ICP-MS.

Results and Discussion

In our previous work, an important accumulation of tin was revealed after 45 h of exposure of the eleutheroembryos [20]. Such content was evaluated by calculating bioconcentration factors (BCFs), following the current guidelines of OECD 305. BCFs are theoretically defined as the ratio of the analyte's concentration at the organisms tested and the exposure solution at steady state [22]. Based on the total content of tin found

in zebrafish eleutheroembryos, the main objective of this paper was to evaluate the likely transformation and/or degradation of TBT.

Extraction and Recovery of Study Species

The extraction of OTCs from biological samples is a critical step when aiming to achieve quantitative recovery and prevent conversion of the original species. The low stability of OTCs and their strong interaction with the matrix makes their extraction from solid samples a very difficult task. Low extraction efficiency as well as the potential losses in the different analytical steps may lead to underestimation of the concentration of the analytes. Thus, rigorous control of the extraction efficiency is necessary. Combination of an organic solvent, of low to medium polarity, with an acid (e.g., acetic acid or hydrochloric acid) is used in more than 50 % of the reported procedures for OTC extraction [23–25]. All these studies conclude that acidic conditions enhance the extraction efficiency of these compounds yielding high recoveries.

In our study, the very small sample amount (~20 mg) and the high lipid content required a careful analytical optimization of the method in order to achieve high compound extraction recoveries. The lack of reference materials regarding OTC extraction procedures from eleutheroembryos, led us to check the efficiency of our method, performing extractions with oyster tissue (T37, candidate reference material with indicative values for OTCs species). Several acetic acid (HAc) and methanol (MeOH) ratios were tested. The extract was then sonicated, derivatized, re-extracted into hexane, and injected into the GC-FPD, following the parameters described in the "Experimental" section. The best extraction efficiency was obtained by mixing HAc:MeOH in a 1:1 ratio. The results for two HAc:MeOH ratios are shown in Fig. 1. Approximately 80 % recovery was obtained for TBT and DBT in a single step; for MBT, the recoveries were much lower. Consequently, two consecutive and identical steps (n=2) were done to achieve quantitative recovery.

The optimized procedure was used with the eleutheroembryos samples. The results of the extractions are shown in Fig. 2. Only two species, TBT and MBT, were clearly identified and quantified. A peak corresponding to inorganic tin was also observed, but it was significantly broad, making its quantification impossible. Again, MBT appeared as the most difficult species to extract, with 50 % in comparison with the 80 % TBT in the first step. It seems MBT is bound more strongly to the biota in comparison to TBT. This is well known and has been reported by several authors; more aggressive operating conditions or using ultrasonic or microwave extraction procedures are required for MBT extraction [26, 27]. Other studies have proposed the use of chelating agents and strong acidic conditions for MBT extraction [28, 29].

Transformation of TBT by Zebrafish Eleutheroembryos

We have showed high accumulation of TBT during the absorption phase, reaching a maximum value after 45–48 post-absorption (BCF=1280), and a 30 % decrease of tin concentration during the depuration phase [19]. A set of samples equally exposed to TBT have now been analyzed in order to measure TBT and/or its possible degradation compounds.

Figure 3 represents the comparison of the three analytical techniques mentioned above: GC-FPD (as sum of the species), ZFG-AAS (total tin), and ICP/MS (total tin). Results fitted quite satisfactory within among the three methods compared. The high uncertainty observed in some cases is probably due to the different behavior of living

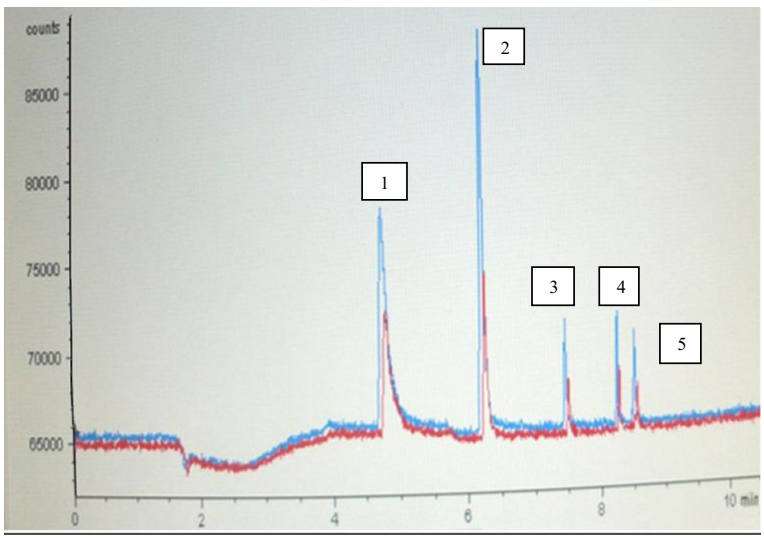

Fig. 1 Chromatogram obtained for the different OTCs from T37 candidate reference material using two extraction mixtures. Compounds: (*1*) monobutyltin; (*2*) tripropyltin (internal standard); (*3*) dibutyltin; (*4*) tributyltin; (*5*) tripheyltin. Acetic acid/water/methanol (1:1:1) (*red line*); acetic acid/methanol (1:1) (*blue line*). Ultrasonic extraction, 60 s; 50 % amplitude. Derivatization with $NaBEt_4$. Re-extraction into hexane

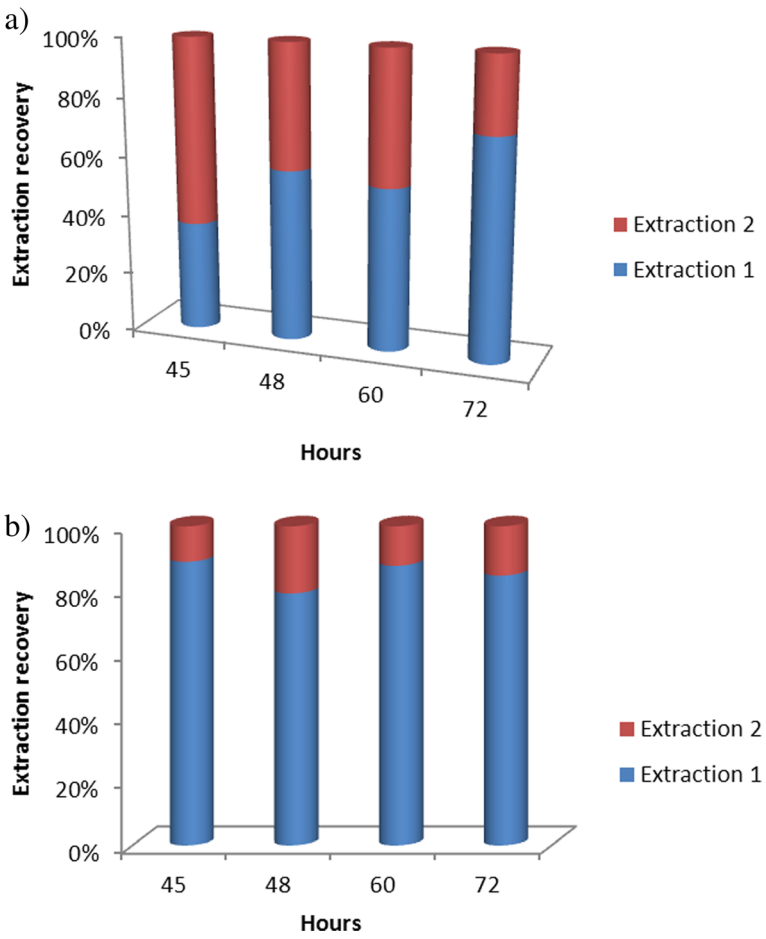

Fig. 2 Evaluation and optimization of the extraction efficiency in two steps from zebrafish eleutheroembryos for **a** MBT and **b** TBT. Extraction mixture: acetic acid/methanol (1:1). Ultrasonic extraction, 60 s; 50 % amplitude. Derivatization with $NaBEt_4$. Re-extraction into hexane; the *X*-axis represents the different hours for the absorption (45 and 48) and depuration phase (60 and 72)

organisms: each replicate is composed of 15–20 eleutheroembryos. A decrease of total tin content was observed over time, either expressed as total tin (GFAAS and ICP/MS) or as the sum of OTCs (GC-FPD). The decrease is more pronounced during the depuration phase (60 and 72 h), implying a transformation process by dealkylation of the most toxic species, TBT, into the less toxic DBT and/or MBT. The transformation of TBT into DBT and MBT in

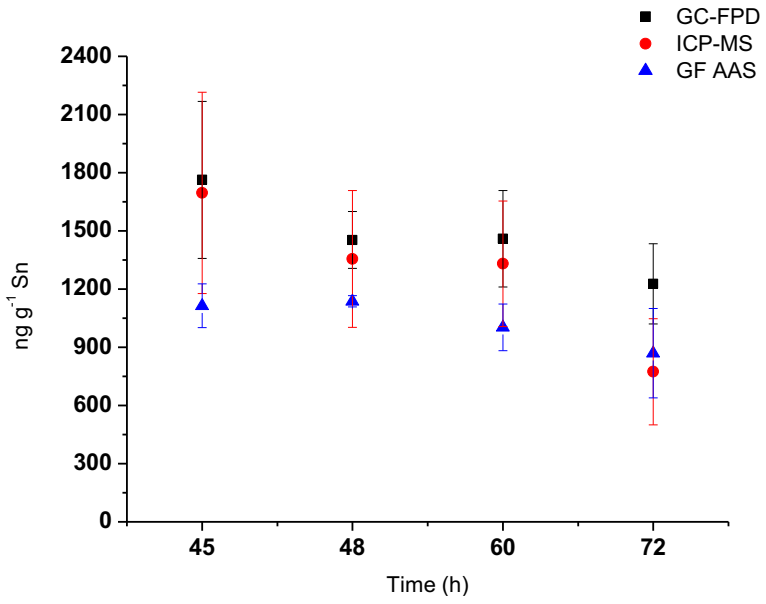

Fig. 3 Total tin quantification in zebrafish eleutheroembryos extracts by using: **a** GC-FPD (sum of organotin species); **b** ICP-MS; **c** ZGF-AAS. The *X*-axis represents the different hours for the absorption (45 and 48) and depuration phase (60 and 72); *N*=3

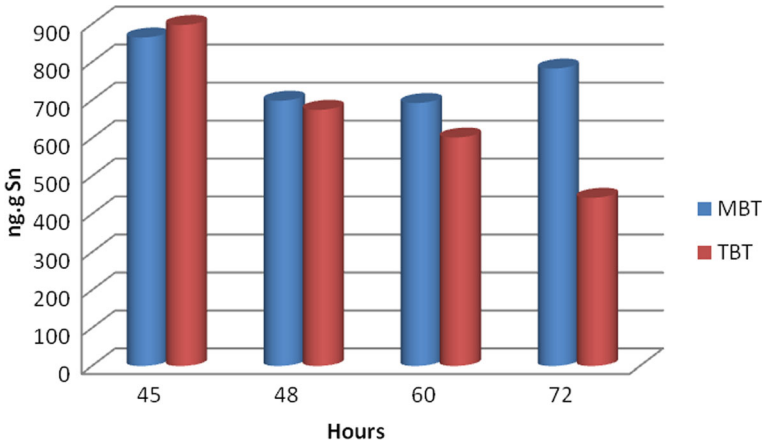

Fig. 4 TBT and MBT concentration changes in zebrafish eleutheroembryos. The *X*-axis represents the different hours for the absorption (45 and 48) and depuration phase (60 and 72); *N*=3

biota samples has been previously reported [30]. TBT and its main degradation products, DBT and MBT, have been detected in different environmental compartments, both in marine [31–33] and terrestrial [34, 35] systems. The occurrence of the less toxic compounds in the environment has so far been related to the degradation of TBT caused by microbial activity and/or photochemical reactions, but some evidence for direct input of MBT and DBT has also been found [3]. Some authors have recently determined residue levels of organotin compounds in five species of deep-sea fish, indicating a certain ability of fish to transform TBT, in contrast to triphenyltin, which preferentially accumulates in the liver [36]. TBT is accumulated in large amounts in animals from lower trophic levels, because they have a low capacity to degrade this compound. As an example, Souza et al. [37] found a good correlation between TBT and its metabolites in marine mussels, indicating that most of the DBT and MBT in mussel tissues are derived from TBT. Furthermore, this biotransformation can be very fast [38].

This well-documented transformation was also observed in our samples. MBT and TBT concentrations measured in the eleutherioembryos during the absorption and depuration phases are shown in Fig. 4 and Table 1. At 45 h, when a steady state was reached in the bioconcentration experiment, MBT and TBT concentrations were practically identical. From then MBT starts to increase whereas TBT decreases. It is noteworthy to indicate that TBT is transformed directly into MBT, with no DBT being detected, neither during absorption nor the depuration phase. This finding is not very common as DBT is generally found as the first degradation compound of TBT. However, a recent study of accumulation and transformation in *Thais clavigera* supports our results [39]. The study showed that TBT rapidly accumulated in the digestive and reproductive organs, and was rapidly eliminated and biotransformed. MBT was the primary metabolite in all tissues, indicating a significant metabolism of TBT by the whelks. The absence of DBT could indicate a rapid TBT detoxification mechanism in eleutheroembryos. This could be related with the fast bioaccumulation kinetics of TBT shown in our previous work [19]. On the other hand, the decreasing rate of TBT is not proportional to the increasing rate observed for MBT, which could be due to detoxification and/or conversion to inorganic tin but this fact can only be affirmed qualitatively. Regarding the depuration phase, we have established that after 24 h of depuration, there is a significant fraction of TBT that is not excreted (~40 %), which is in agreement with the highly persistent nature of this pollutant.

Table 1 Mean concentration±uncertainty of TBT and MBT determined for zebrafish eleutheroembryos

Organotin (ng g^{-1})				
Time (h)	MBT	*U*	TBT	*U*
45	898	263	865	365
48	699	100	675	248
60	693	150	602	280
72	783	235	443	183

Conclusions

This work provides further information on zebrafish eleutheroembryos detoxification exposed to TBT. The results suggest a rapid conversion of the high amount of TBT accumulated by these organisms into less toxic species, MBT, and likely inorganic tin. The absence of DBT could be attributed to fast biotransformation kinetics. Still, an important amount of TBT remains in zebrafish eleutheroembryos due to its highly persistent character.

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