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Running title: Reproductive impacts of TBT and TPT on *Lymnaea stagnalis*

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Abstract: Tributyltin (TBT) and triphenyltin (TPT) are emblematic endocrine disruptors, which have been mostly studied in gonochoric prosobranchs. Although both compounds can simultaneously occur in the environment, they have mainly been tested separately for their effects on snail reproduction. Because large discrepancies in experimental conditions occurred in these tests, the present study aimed at comparing the relative toxicity of TBT and TPT under similar laboratory conditions in the 0–600 ng Sn/L range. Tests were performed on the simultaneous hermaphrodite *Lymnaea stagnalis,* a freshwater snail in which effects of TPT were unknown. Survival, shell length and reproduction were monitored in a 21d semi-static test. Frequency of abnormal eggs was assessed as an additional endpoint. TPT hampered survival while TBT did not. Major effects on shell solidity and reproduction were observed for both compounds, reproductive outputs being more severely hampered by TBT than by TPT. Considering the frequency of abnormal eggs allowed increasing test sensitivity, since snail responses to TBT could be detected at concentrations as low as 19 ng Sn/L. However, the putative mode of action of the two compounds could not be deduced from the structure of the molecules or from the response of apical endpoints. Sensitivity of *L. stagnalis* to TBT and TPT was compared to the sensitivity of prosobranch molluscs with different habitats and different reproductive strategies.

Keywords: Reproductive toxicity, Endocrine Disruptors, Mollusk toxicology, Organotin, Toxicity Mechanisms

INTRODUCTION

Organotins have been used for more than 50 years mainly as pesticides and antifungal agents, the widely used tributyltin (TBT) and triphenyltin (TPT) being the active biocides of antifouling paints [1]. Their use in antifouling paints has been prohibited in 2008 [2], but residues can still be found both in marine environments and in freshwater ecosystems, at concentrations up to e.g. 7.1 µg TBT/L [3]. Therefore, the assessment of toxic effects of these compounds to aquatic, freshwater wildlife is still relevant. To date, the effects of TBT and TPT have mostly been investigated separately as single compounds. These studies pointed out some similarities between TPT and TBT regarding their physico-chemical properties and biological effects. Indeed, these molecules have a closely related structure: a tetravalent tin core with either three butyls (TBT) or three phenyls (TPT). These compounds are fairly persistent in the environment, being retained in the sediments [4-6]. Furthermore, they penetrate biological systems and can be stored at high concentrations in lipid-rich tissues of aquatic organisms e.g. TBT levels up to 233 ng/g have been recorded in retail mollusc products [7]. TBT and TPT accumulate preferentially in the hepatopancreas and kidneys and at lower levels in the heart and brain [8-10]. Consequently, both TBT and TPT can induce adverse effects on wildlife, which have mainly been studied in marine molluscs.

As early as 1975, adverse impacts of TBT were observed in the oyster *Crassostrea gigas*, shell calcification in adults leading to stunted growth [11]. At low concentrations (ng/L range), TBT was shown to induce the imposition of male sex organs in female prosobranchs [12]. This phenomenon, named *imposex*, has now been reported to occur in more than 200 mesogastropods and neogastropods species [13], in both marine and freshwater species. At highly contaminated sites, females can be sterilized or even killed, which may affect population dynamics [14]. TPT was also shown to induce reproductive failure through *imposex* in different gastropod species [15-17]. A number of studies

reported other types of reproductive alterations in freshwater species due to exposure to organotins [18-24]. For instance, exposure of *Lymnaea stagnalis* to TBT at 1 µg Sn/L was shown to induce abnormal development of embryos (absence of shell), and a decrease in egg-hatchability; exposure to 10 µg Sn/L led to a complete hatching failure [19].

In the environment, organotins generally occur as mixtures of TBT and TPT (and their derivatives *i.e.* DBT, MBT, DPT and MPT) [25, 26]. Therefore, a growing number of studies are devoted to the study of the relative toxicity of these compounds, which constitutes the first step towards the study of mixture toxicity. Comparisons of bioaccumulation and biological responses to TBT and TPT have already been done in several gastropod species *e.g.* the muricids *Thais clavigera* and *Thais bronni* [27] and *Bolinus brandaris* and *Hexaplex trunculus* [28]. These studies allow a straightforward comparison of TBT and TPT effects under similar test design and experimental conditions and using similar test endpoints. This ensures both quantitative and qualitative reliability of effect comparisons though the avoidance of experimental confounding factors, which often occur when comparing data from different studies. To date, these comparative studies only dealt with gonochoric species, where toxic effects can be assessed through direct monitoring of the morphological changes in the sexual apparatus. Furthermore, impacts on endocrinology and especially on sexual steroids are more easily understood in species which exhibit separated genders, where the male and female hormonal reproductive pathways can be distinguished. Effects on the reproductive pathways and performances in hermaphroditic species are more subtle. Probably due to the complex sexual apparatus and the variety of reproduction strategies in simultaneous hermaphrodite gastropods (e.g. selfing *vs*. outcrossing) [29], effects of organotins in these animals have not been extensively investigated so far [20]. In particular, it is important to investigate suitable endpoints allowing to highlight reproductive effects in these species where sex-differentiation cannot be used as an effect criterion. To date, most studies have been conducted using the great pond snail *Lymnaea stagnali*s (Linné, 1758). This holarctic freshwater snail lives in ponds and

lakes. It is a simultaneous hermaphrodite, which can outcross and self-fertilize [30]. It has been identified as one of the most relevant mollusc species for assessing reprotoxic effects of chemicals [20, 29, 31-33]. Because its neurohormonal control of reproduction is reasonably well understood compared to other mollusc species [32-35], and also because it has been shown to be sensitive to endocrine disruptors [19, 21, 24, 31, 35], standard OECD test guidelines for apical reprotoxicity tests (both partial and full life-cycle tests) with *L. stagnalis* are currently under development.

In the present study, we aimed at investigating the reproductive effects of the emblematic organotin compounds TBT and TPT, known as endocrine disruptors in some mollusc species, in the hermaphroditic freshwater snail *L. stagnalis*. Effects of TBT and TPT were studied comparatively under the same controlled laboratory conditions. Reproductive effects were assessed through a set of complementary apical endpoints (i.e. number of egg-clutches, number of eggs, and frequency and type of abnormal eggs). Adult survival and growth were also monitored. Effects of TBT and TPT were compared both qualitatively and quantitatively. Influence of the structure of the molecules on the biological responses and possible corresponding modes of toxic action were discussed. Moreover, biological responses of this hermaphroditic snail to TBT and TPT were compared to available data in other mollusc species.

MATERIAL AND METHODS

Test organisms

Lymnaea stagnalis (Linnaeus, 1758) (Mollusca, Gastropoda, Panpulmonata, Heterobranchia) has been reared at the INRA Experimental Unit of Aquatic Ecology and Ecotoxicology (Rennes, France) under laboratory conditions as previously described [30]. Culture medium consisted in dechlorinated, charcoal-filtered tap water with the following physico-chemical characteristics: pH = 7.7 ± 0.2, conductivity = 623 ± 60 µS/cm, dissolved oxygen = 7.3 ± 2 mg/L and water hardness = 254 ± 7 mg $CaCo_3$/L. Rearing conditions were the following: temperature = 20 ± 1 °C, photoperiod = 14:10 L:D and

light intensity = 155 ± 35 lux. Snails were fed three times per week with organic lettuce. The RENILYS® strain was used. Young adult snails, in which reproduction endpoints are more sensitive to chemicals including some EDCs than in fully-grown snails [35], were used. Snails of homogenous size (22.5 ± 2.5 mm) and age (4 ± 0.5 months) were sampled from the culture and acclimatized to test conditions (*i.e.* similar as culture conditions, but with a higher food quantity provided per individual) during 48 hrs prior to chemical exposure.

Tested chemicals and concentrations

Tributyltin hydride (CAS Number 688-73-3) and triphenyltin chloride (CAS Number 639-58-7) were dissolved in analytical grade acetone (99.9 % purity) in order to prepare stock solutions (10µg/µL). Final solvent concentration (100 µl/L) was homogenous among treatments, as recommended by OECD [36] (except for the water controls). Exposure media consisted in culture water contaminated with stock solutions. Organotin concentrations were chosen based upon literature data in gastropods [15, 18, 22, 37] and *L. stagnalis* [19, 21]. Range finding tests in *L. stagnalis* were also performed under similar tests conditions [38, 39]. Based upon this information, the chosen nominal concentrations were 45, 100, 220, 480 and 1065 ng Sn/L for TBT, and 100, 215, 755, 1000 and 2626 ng Sn/L for TPT. In order to facilitate the quantitative comparison of TBT vs. TPT effects, all concentrations were expressed in tin equivalent: concentrations could thus be compared on a molar basis.

Test design and biological endpoints

Six replicates (each with five snails in 1 L glass beakers) per tested concentration, water control and solvent control were randomly distributed in the exposure room. Snails were exposed to toxicant for only 21 days because this duration was sufficient to evidence effects and assess EC_x values for the tested compounds in the proposed experimental conditions (as determined in pre-experiments). Test water was renewed with freshly contaminated medium in order to maintain exposure concentrations and adequate physico-chemical properties of test water. Renewal rates resulted from a compromise between

maintaining exposure concentration and avoiding to much stress to the snails. Independent studies showed that in out test conditions, TBT water concentration rapidly dropped (92% losses in 72h), so that the TBT test medium was renewed every other day. TPT was more stable (62% losses in 72h), so that the TPT test medium was renewed every three days. Tests were conducted at 20.5 ± 0.6°C and 14:10 Light:Dark photoperiod as previously described [35]. Snails were fed daily ad libitum with organic lettuce rinsed with culture water, which is an adequate food source to support adult snails growth and reproduction [40].

Dead snails were counted and removed daily. Individual shell length was measured using a digital calliper (Mitutoyo Corp, Kanogawa, Japan) at day 0 and 21 to assess a possible impact on growth. Effects on reproduction were estimated through the monitoring of cumulated number of egg-clutches per snail and cumulated number of eggs per snail. Every day, clutches were counted, removed using a sharp-edge spoon, and photographed. The number and quality of eggs was determined based upon observation of the numeric pictures. Egg-quality was assessed by determining the frequency of four types of abnormalities (Fig. 1 a-e): "polyembryonic egg", i.e. the presence of several embryos per egg, "unfertilized egg", i.e. the absence of embryo in the egg, which only consists in the egg-shell and albumen, "atrophied albumen", i.e. damaged egg-shell containing an abnormally low albumen quantity, and "single embryo", i.e. presence of a non-developing embryo, without an egg-shell and without albumen. Polyembryonic and unfertilized eggs have been described before [41-43], and polyembryony has been shown to be a sensitive endpoint [41]. The two others abnormalities are described for the first time in *L. stagnalis*.

Chemical analysis

Water was sampled in controls and every tested concentration at the beginning, mid-term and end of the tests. Water was collected both 15 minutes after stirring stock solutions in clean water (i.e. new exposure water) and just before water renewal (i.e. old exposure water) in order to allow the calculation

of the time weighted average exposure concentrations. For each concentration and sampling date, three samples of 1L were collected, which consisted in a mixture of 165 ml of water extracted from each exposure replicate. In the TBT experiment, mucus that had accumulated onto the walls of the test beakers was also collected for chemical analysis. Samples were frozen until analysis of their mono-, di- and tri-butyltin and triphenyltin content. Analyses were performed by coupled capillary gas chromatography - mass spectrometry (GC-MS-MS), with a limit of quantification of 10 ng Sn/L equivalent [44].

Data analysis

Actual exposure concentrations were calculated as the time-weighted average of measured values over the test, using the formula proposed in [45]. Biological data were analysed using standard statistical analysis procedures, as described in OECD [46], with the Sigma-Stat (Sigma-Stat, Jandel Scientific, CA) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) softwares. The analyses of survival and reproduction data were based upon observations for the six replicates whereas the analysis of growth data was based on individual length measurements. The analysis of abnormality frequency was based upon the total number of collected clutches during the experiment. Statistical differences between water and solvent controls were determined using T-tests or Wilcoxon tests. When a significant difference with water control was shown for one of the compounds, solvent controls were used as the reference in subsequent statistical studies for both compounds. Indeed, using the same type of controls for the calculation of ECx values allows avoiding confounding effects of the solvent. In other cases, water and solvent controls were combined. Differences among treatments in survival, shell length and cumulated number of clutches and eggs per individual were tested using Kruskal-Wallis tests, with Dunn's post-hoc tests for survival, size and with Dunnet's post hoc test for reproductive endpoints. Differences in the frequency of abnormal eggs were assessed using Mann-Whitney tests. All tests were performed with $\alpha = 0.05$. In case of significant effects of TBT or TPT, LCx or ECx were calculated

using a logistic regression model [47]. The 95% confidence intervals were simulated based upon weighted residues in order to account for differences in variance across treatments and using 5000 bootstrap simulations. The Microsoft Excel macro REGTOX_EV7.0.6 xls was used for this purpose [48].

RESULTS

Actual exposure conditions

Chemical preparation and contamination method resulted in exposure concentrations corresponding to 62.7 ± 14.2 % and 87.5 ± 17.6 % of the nominal TBT and TPT concentrations, respectively, at day 0. Water concentrations dropped rapidly and with different kinetics for TBT and TPT. Before water renewal, exposure concentrations had dropped to 35.7 ± 4 % and 18.1 ± 3 % of the nominal TBT and TPT concentrations, respectively. This resulted in time-weighted average concentration ranges of 19, 43, 94, 197 and 473 ng Sn/L for TBT and 45, 74, 187, 265 and 590 ng Sn/L for TPT. High TBT concentrations were also found in the mucus sampled after 21 days (e.g. 1563 ± 638 ng Sn/L at the highest tested concentration). Both mono- and di-butyltin were found in water due to the degradation of TBT. MBT and DBT concentrations were not included in the calculation of TBT time-weighted average concentrations because their concentration were generally lower than the quantification limit, so that they could be neglected.

Survival

Survival was 100% in water controls from both tests and 93% and 88% in the solvent controls from TBT and TPT tests, respectively. No significant mortality was recorded following exposure to TBT over the test duration. Exposure to the highest tested concentration of TPT (590 ng Sn/L) led to 100% mortality after three days of exposure ($p < 0.05$, Dunn's post hoc test), while lower concentrations did

not significantly affect survival. The corresponding LC50-21d was estimated at 436.1 [308.1 – 433.6] ng Sn/L.

Shell size and integrity

Shell length at 21 days was significantly reduced in snails exposed to TBT concentrations exceeding 94 ng Sn/L (Fig. 2a). A non significant decrease was also observed in snails exposed to 43 ng Sn/L. A careful inspection of snails showed that the apex of the shells was broken in some to all exposed individuals, leading a reduced shell length. Snails exposed to 0 and 19 ng Sn/L generally did not suffer from such injury. This suggests that broken shell were not an experimental artefact, but a consequence of exposure to TBT. Indeed, the frequency of harmed shells increased linearly with TBT concentration ($R^2 = 0.94$) and reached 100% at the highest tested concentration of 473 ng Sn/L (Fig. 3a). Therefore, observed reduction in shell length provide evidence for effects of TBT on the shell solidity. Analyzing size data only in snails which did not suffer shell injury allowed to highlight a significant reduction in growth for concentrations exceeding 94 ng Sn/L. As the growth effect was not very intense in the tested concentration range, no reliable ECx value could be calculated. Damaged shells were observed at all tested TPT concentrations (but not in controls), suggesting that TPT also had an impact on shell solidity (Fig. 3b). Damage frequency was comprised between 50 and 70% whatever the exposure concentration. TPT had no significant effect on growth (Fig. 2b).

Egg-laying behaviour

The time-course of effect was different in snails exposed TBT concentrations lower or equal to 94 ng Sn/L, where egg-laying regularly occurred but at a slower rate than in controls, *vs* in snails exposed to 197 and 473 ng Sn/L, where egg-laying ceased after one week of exposure. The cumulated number of clutches produced per individual over 21 days decreased in all snails exposed to TBT as compared to the water controls, from -24% at 19 ng Sn/L to -96% at 473 ng Sn/L (Fig. 4a). A significant difference with solvent controls in the number of produced clutches ($p < 0.05$, Dunnett's post hoc test) was detected

from 94 ng Sn/L. Exposure to the two highest TBT concentrations resulted in a severe reduction in egg-laying activity ($p < 0.001$, Dunnett's post hoc test). The corresponding EC50-21d was estimated at 118.3 [99.7 – 171.9] ng Sn/L.

In the TPT test, the time-course of effect was similar in snails exposed to all tested concentrations; snails regularly laid eggs but the egg-laying rate was lower than in controls. Egg-laying activity was significantly lower in solvent controls ($p < 0.05$, Wilcoxon rank sum test) than in water controls (Fig. 4b). Solvent control was thus used as reference for statistical tests for both TPT and TBT. Exposures to 265 and 590 ng Sn/L caused a significant decrease ($p < 0.01$ and $p < 0.001$, respectively, Dunnett's post hoc test) of the cumulated number of produced clutches (-38% and -81% of the solvent control value, respectively). Based upon these results, the estimated EC50-21d value was 264.1 [258.5 – 280.5] ng Sn/L.

Fecundity

The effect patterns and estimated NOEC and LOEC values of TBT and TPT on fecundity were similar to the patterns observed for egg-laying behaviour: the LOEC values were 94 and 264 ng Sn/L ($p < 0.01$ and $p < 0.001$, respectively, Dunnett's post hoc test) for TBT and TPT, respectively.

Compared to water controls, a non-significant reduction of fecundity was already observed at the two lowest TBT concentrations, and was particularly severe at the two highest tested concentrations (from -32% at 19 ng Sn/L to -97% at 473 ng Sn/L, as shown in Figure 5a). The corresponding EC50-21d value was 106.2 [84.4 – 125.6] ng Sn/L, which was not significantly different from the EC50-21d found using oviposition as an endpoint.

A significant solvent effect on fecundity was again observed in the TPT test ($p < 0.01$, Wilcoxon rank sum test). Fecundity of the snails exposed to the two highest TPT concentrations (265 and 590 ng Sn/L) was significantly reduced (-36% and -85% of the solvent control value, respectively) as shown in

Figure 5b. The corresponding EC50-21d value was 263.9 [253.8 – 280.5] ng Sn/L, which was similar to the EC50-21d found for oviposition.

Egg-abnormalities

Frequency of abnormal eggs per clutch increased over the test duration. After 21 days, it was significantly higher in snails exposed to TBT than in controls even at the lowest tested concentration of 19 ng Sn/L ($p < 0,05$ Dunnett's post hoc test). Effects of TBT on the frequency of unfertilized eggs, eggs with atrophied albumen and single embryo were not significant. Frequency of polyembryony was the most interesting endpoint: it represented 65 % to 100% of observed abnormalities in the lowest and highest tested concentrations, respectively. It increased from +80% at 19 ng Sn/L to +177% at 473 ng Sn/L when compared to the controls (Fig. 6). This increase was significant for all tested concentrations ($p < 0.05$, Dunnett's post hoc test). Corresponding EC50-21d was 23.7 [2.2 – 189.4] ng Sn/L. All eggs produced by parents exposed to 473 ng Sn/L exhibited polyembryony. Alternatively, TPT induced no significant effects on the viability of eggs produced by exposed adults at the tested concentration range.

DISCUSSION

Chronic effects of organotins in L. stagnalis

TBT metabolite concentrations were very low (*i.e.* below the quantification limit), which suggests that degradation might not be the process which mostly contributed to the decrease in TBT concentration in water. Losses were probably mostly due to adsorption in e.g. the mucus. Indeed, high TBT concentrations were found in the mucus sampled after 21 days i.e. three to five-fold higher concentrations than the measured peak concentration in water. TBT content in mucus resulted most likely from direct adsorption from water after the mucus has been released and possibly, to a lesser extend, to elimination of TBT by snails *via* the mucus. This adsorption might partly explain why only 62.5% of the targeted nominal concentrations were found in the water samples. It highlights the need to

wipe-off mucus from test beakers between water renewals in order to limit adsorption of TBT on the test chamber walls, which reduces its availability to the snails.

Exposure of *L. stagnalis* for 21 days to TPT in the concentration range 45–590 ng Sn/L induced mortality at the highest tested concentration (LC50-21d = 436.1 ng Sn/L), and a variety of sublethal effects at lower concentrations, i.e. decrease in the solidity of the shell at all tested concentrations, decrease in the egg-laying activity and fecundity, these endpoints leading to an identical $EC_{50\text{-}21d}$ value of 264 ng Sn/L. TPT did not affect egg-quality at the tested concentration range.

A 21d exposure of *L. stagnalis* to TBT in the range 19–473 ng Sn/L did not result in a significant mortality in our study. Alternatively, Segner et al. [24] showed that adult survival was significantly reduced by TBT exposure, with a LC50-21d of 290 ng Sn/L (nominal concentration), which contradicts both the present result and longer term studies from other authors. Indeed, no significant mortality was observed after 49-d and 84-d of exposure to 100 ng Sn/L (nominal concentration [21], as well as after 56d of exposure to the concentration range 7–181 ng Sn/L (unpublished data from a round-robin test). At this concentration range, significant mortality was only observed in a 170-d study where snails were exposed to 410 ng Sn/L [19].

Exposure to TBT induced a decrease in growth at concentration exceeding 94 ng Sn/L. It also induced a decrease in the solidity of the shell in snails exposed to concentrations exceeding 43 ng Sn/L, which was also observed in 21-d tests for nominal concentrations exceeding 94 ng Sn/L by Segner et al. [24]. Exposure to TBT also induced a significant decrease in the egg-laying activity and fecundity of snails exposed to concentrations exceeding 45 ng Sn/L. This is consistent with previous results from Czech et al. [21] who observed a significant decrease of egg-laying at 100 ng Sn/L (nominal concentrations) in a 49-d experiment. Overall, these data confirm the possible occurrence of reproductive effects in *L. stagnalis* exposed to TBT at environmentally relevant concentrations. Results obtained in our partial-life cycle tests are in accordance with results obtained in other partial life-cycle

tests [21, 24] but differ from results obtained in the 170-d full life-cycle test published by Leung et al. [19], where animal were exposed from the embryonic stage to adulthood. In the study of Leung et al., adult fecundity was significantly modified at 41 ng Sn/L (NOEC), which was also the case in our study (NOEC = 43 ng Sn/L). Yet, the effect pattern was quite different (S-Shape dose-response curve in our study *vs.* Inverted-U-shape in Leung's et al. study, with an increase of fecundity at 41 ng Sn/L compared to controls). The magnitude of effects at 410 ng Sn/L was also different. Indeed, the fecundity was reduced by a factor of 10 when compared to control in Leung's et al. study whereas it was reduced by a factor 100 in our study. These results suggest that the test design might greatly influence the biological responses of *L. stagnalis*. Further studies are needed to assess the influence of test duration on the endpoint values in partial life-cycle tests and compare the sensitivity of partial *vs.* full life cycle test with *L. stagnalis*.

Egg-quality was altered by TBT, which was best evidenced through the frequency of polyembryonic eggs in clutches from exposed parents. The increase in polyembryony frequency was already significant at concentrations that were lower than those affecting egg production by adults. This indicates that polyembryony is more sensitive to TBT than the other reproductive endpoints tested (*i.e.* oviposition and fecundity which are the common endpoints in reprotoxicity tests with snails), thus confirming previous findings with acetone [41]. Test endpoints/durations/protocols vary a lot among published studies on the effects of TBT in *L. stagnalis*, leading to a large variability in published NOEC values for some endpoints. This variability is also due to the lack of data on actual exposure concentrations in most published papers, which hampers a sound comparison of the published results. In this respect, the forthcoming standardization of reproductive toxicity test protocols with *L. stagnalis* will help in deriving more reliable conclusions on the toxicity of TBT, TPT and other types of chemicals.

Comparison of the responses of L. stagnalis to TBT vs. TPT

Differences in the sensitivity of *L. stagnalis* to similar concentration ranges of TBT and TPT were

observed for most of the studied endpoints. The tested TBT concentrations (up to 473 ng Sn/L) had no significant effect on survival whereas 100%, mortality was observed at the highest tested TPT concentration (590 ng Sn/L), which suggests a higher toxicity of TPT than of TBT to *L. stagnalis*. Results from a pre-test with TBT confirmed that TBT has no effect on survival at 590 ng Sn/L (the NOEC in this pre-test was 627 ng Sn/L, while the LOEC was 6,270 ng Sn/L where 100% mortality occurred at the end of the test, data not shown). TBT had a significant effect on snail's growth, while no effect occurred due to TPT exposure. Because both TBT and TPT led to shell injuries, shell size might not be the most reliable growth indicator when assessing organotins effects in *L. stagnalis*. Indeed, a decreased of shell size due to an injury can be wrongly interpreted as an effect on growth. In order to avoid such misinterpretation, we recommend not using length data from individuals with broken shells for the statistical analysis of growth effects. Discarding such individuals from the analysis might lead to the loss of a number of data and thus to a reduced statistical power when testing effects on growth (*e.g.* in the present study, 100% of snails were injured at the highest TBT concentration). Growth effects should rather be assessed through measurement of the soft body dry weight in order to avoid growth effects misinterpretation and maintain a sufficient statistical power. It is likely that the decrease in shell solidity was due to decalcification, which has already been observed for TBT in *L. stagnalis* [24] and other molluscs (e.g. in oysters [49]). Similarly, tri-alkylated tin compounds have been shown to interfere with calcification processes in mammals [49], which might also apply to *L. stagnalis* exposed to TPT.

Both compounds significantly reduced egg-laying and fecundity, but effective concentrations were lower for TBT than for TPT, as reflected by the $EC_{50\text{-}21d}$ values for reproductive endpoints. EC50-21d based on fecundity data were equivalent to values obtained from oviposition data for TBT (EC50-21d values of 106 and 118 ng Sn/L, respectively – no significant difference as assessed through the overlapping of 95% confidence intervals) and for TPT (with a similar EC50-21d value of 264 ng Sn/L and no overlapping of confidence intervals). These results suggest that both reproductive endpoints have

a similar sensitivity to TPT and TBT in this particular experimental setup, although oviposition and fecundity are known to be under the control of two different hormonal pathways in *L. stagnalis* [32]. Exposure to TPT did not affect egg-quality, whereas polyembryony often occurred in eggs produced by TBT-exposed snails. Polyembryony thus exhibited a different responsiveness to TBT and to TPT and was the most sensitive endpoint in the TBT test. The use of acetone as the carrier solvent did not significantly influence snail reproduction or egg-quality in the TBT test but did hamper significantly all reproductive performances in the TPT test. Previous in-house studies showed that acetone reproductive effects occur from time to time in our experimental conditions. Reasons for this remain unclear; this may be due to e.g. difference in solvent purity or snail sensitivity from one experiment to the other. This highlights the need for additional studies devoted to the assessment of solvent effects in juveniles and adults of *L. stagnalis*, in order to complement the recently published results on solvent effects in embryos [43].

Reasons for differences in sensitivity of *L. stagnalis* to TBT *vs.* TPT remain to be elucidated. Differences in molecular structure of these organotins might be a relevant explanation. Indeed, it is assumed that toxicity of organotins is more influenced by the alkyl substitutes than by the anionic substitute [6]. A recent study in *Mytilus edulis* confirmed that alkylation of organotins influence their toxicity to molluscs [50]. Therefore, differences in the responses of *L. stagnalis* to TPT *vs.* TBT is likely to be related to differences in their alkylation. However, it is not know how such differences in the structure of the molecules might lead to different modes of action of these compounds in *L. stagnalis*. Previous studies highlighted the possible endocrine effects of TBT and TPT in gastropods [51-55]. Hormonal pathways involved in the response of various gastropods to TBT were investigated, focusing on the mechanisms of *imposex* induction in gonochoric marine species [51, 54, 56-58]. These studies highlighted that *imposex* is related to an alteration of steroid homeostasis, mediated by the inhibition of enzymes such as cytochrome-P450 aromatase or acyltransferase that are involved in steroid biosynthetic

pathways [53, 57, 59]. Other *in vivo* and *in vitro* studies showed that TBT and TPT are potent activators of nuclear receptors such as the retinoid X-receptors (RXR), leading to transcription of genes involved in steroid homeostasis [52, 60-63]. Other studies highlighted effects of TBT and TPT on different components of the microsomal monooxygenase system of bivalves, *Mytilus galloprovincialis* and *Ruditapes decussata*, and of the gastropod *Thais haemastoma* [64]. At last, Lyssimachou *et al.* [15] showed the potency of TPT to alter lipid metabolism in females of the ramshorn snail *Marisa cornuarietis*. However, it is not known to which extent these findings can be extrapolated to hermaphroditic species like *L. stagnalis*. In the present study, reproductive effects of TPT occurred at quite high concentrations (i.e. $LC_{50\text{-}21d}$ was only two-fold higher than the reproductive EC50-21d). EC50-21d were similar when estimated using fecundity or egg-laying data, indicating no relationship between the hormonal pathways involved in the control of these processes and the biological response. Furthermore, no significant increase in the frequency of abnormality occurred in the offspring of exposed snails. Therefore, results of the apical reproduction test suggest that the observed reproductive impacts are probably linked to the toxicity of TPT to the snails rather than being a consequence of endocrine disruption. Alternatively, TBT concentrations that induced reproductive effects in *L. stagnalis* were much lower than the lethal concentrations reported in other studies conducted with this species [19, 24], and occurred at environmentally relevant concentrations. In addition, the frequency of polyembryony in the offspring increased in exposed snails. These results suggest that TBT might act as an endocrine disruptor in *L. stagnalis* too. Even if apical endpoints might bring clues on possible modes of action of TBT and TPT in *L. stagnalis*, studies should be implemented to check to what extent and for which compounds the observed deleterious effects of organotins are actually due to endocrine disruption [31-32].

Comparison of responses of the hermaphroditic snail vs gonochoric species

Existing data, including the present results, showed that TPT and TBT have different acute and

chronic effects in various mollusc species. For instance, exposure of the parthenogenetic snail *Potamopyrgus antipodarum* to TBT and TPT in sediment biotests led to a significant reduction of the reproductive output at environmentally relevant concentrations [8, 65]. Effect concentrations were lower and the intensities of effects on reproduction were higher in mud snails exposed to TPT as compared to those exposed to TBT. Alternatively, acute toxicity occurred in snails exposed to TBT but not in those exposed to TPT. Based upon these results, the effects of TPT and TBT in *P. antipodarum* are just opposite to the alterations we highlighted in *L. stagnalis*. In another study, it was shown that females of the rock shell *T. clavigera* had an similar sensitivity to TBT and TPT (via direct injection in soft tissues), while males were more sensitive to TPT than to TBT [17]. Interestingly, it appears that three prosobranch snails with different habitats (fresh-, brackish- and marine waters) and different reproductive strategies (parthenogenesis, hermaphrodism and gonochorism) were all responsive to organotin compounds, but their sensitivities were species- and sex-dependant, as already reviewed by [66]. Additional studies are required to provide explanations for differences in responsiveness and sensitivity to TBT and TPT in and between species.

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CAPTION OF FIGURES

Figure 1: Different abnormalities of egg-quality observed in *Lymnaea stagnalis* (A) Normal egg; (B) Polyembryonic egg; (C) Unfertilized egg; (D) Egg with atrophied albumen; (E) Single embryonic cell.

Figure 2: Mean shell size after a 21d exposure to (A) TBT or (B) TPT. Error bars stand for SD over six replicates (*: $p<0.05$).

Figure 3: Frequency of broken shells observed over six replicates after a 21d exposure to (A) TBT or (B) TPT.

Figure 4: Mean cumulated number of egg-clutches laid per individual after a 21d exposure to (A) TBT or (B) TPT. Error bars stand for SD over six replicates (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Figure 5: Mean cumulated number of eggs laid per individual after a 21d exposure to (A) TBT or (B) TPT. Error bars stand for SD over six replicates (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Figure 6: Frequency of the polyembryonic eggs (among the total number of abnormal eggs) found during a 21 d exposure to TBT. Error bars stand for SD over all produced abnormal eggs (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

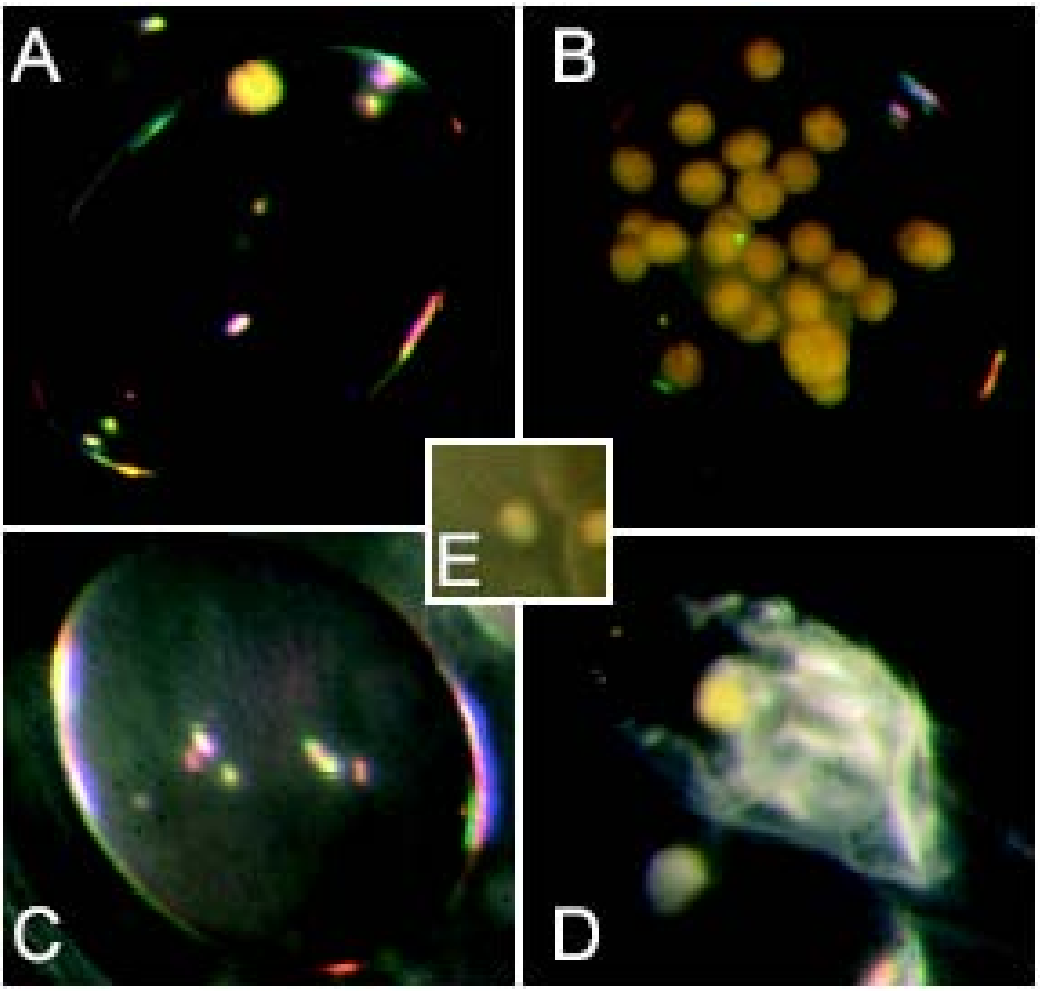

Figure 1

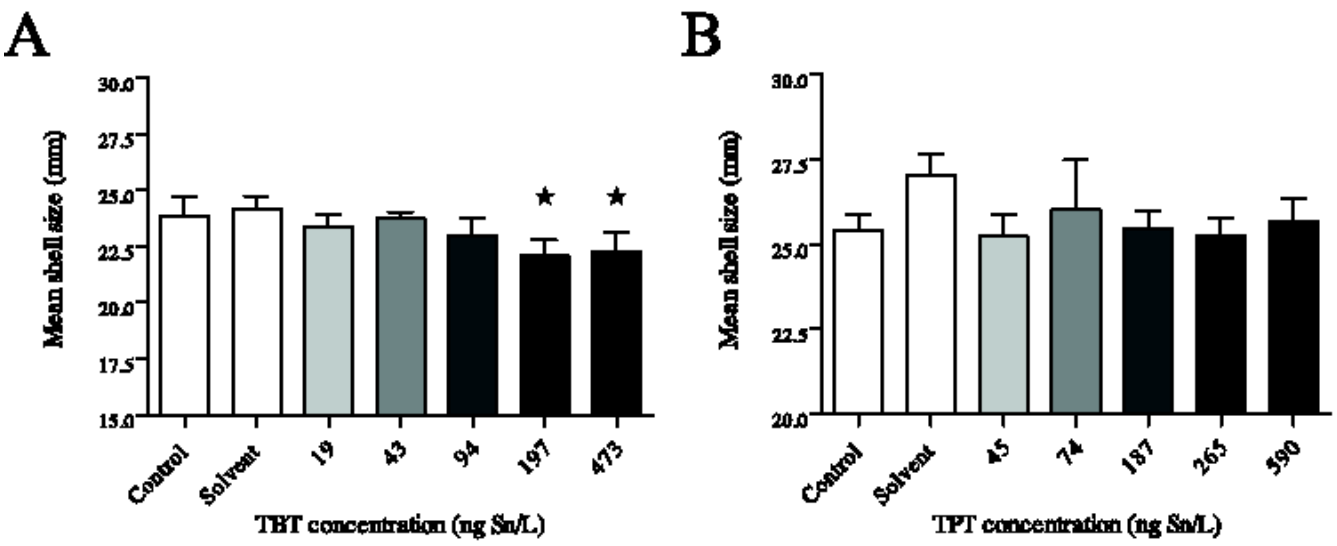

Figure 2

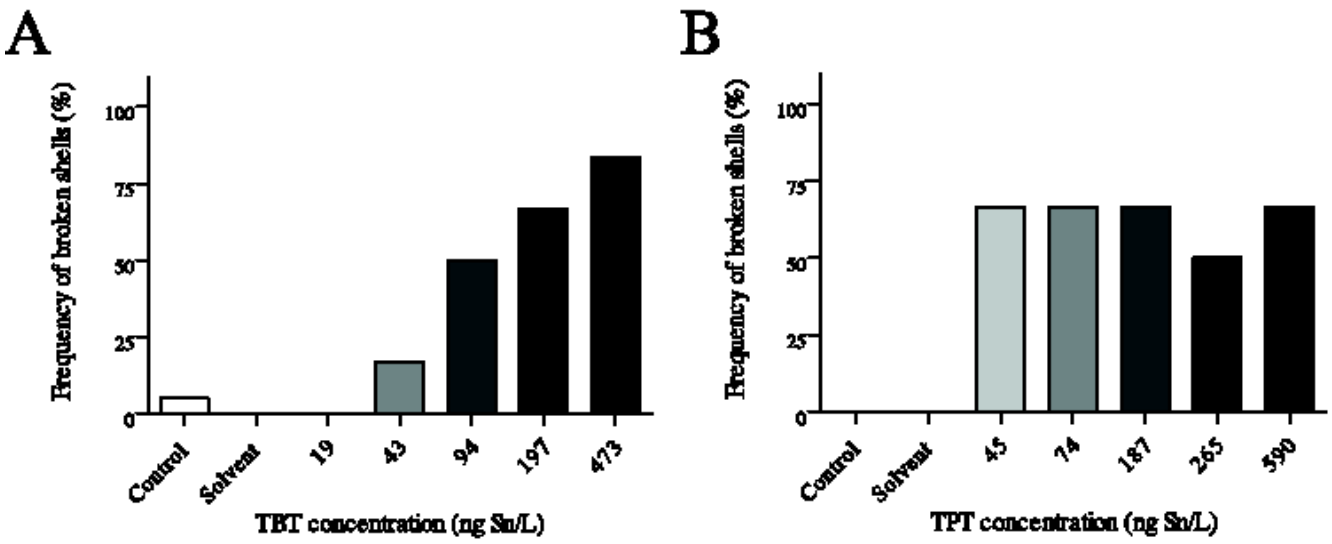

Figure 3

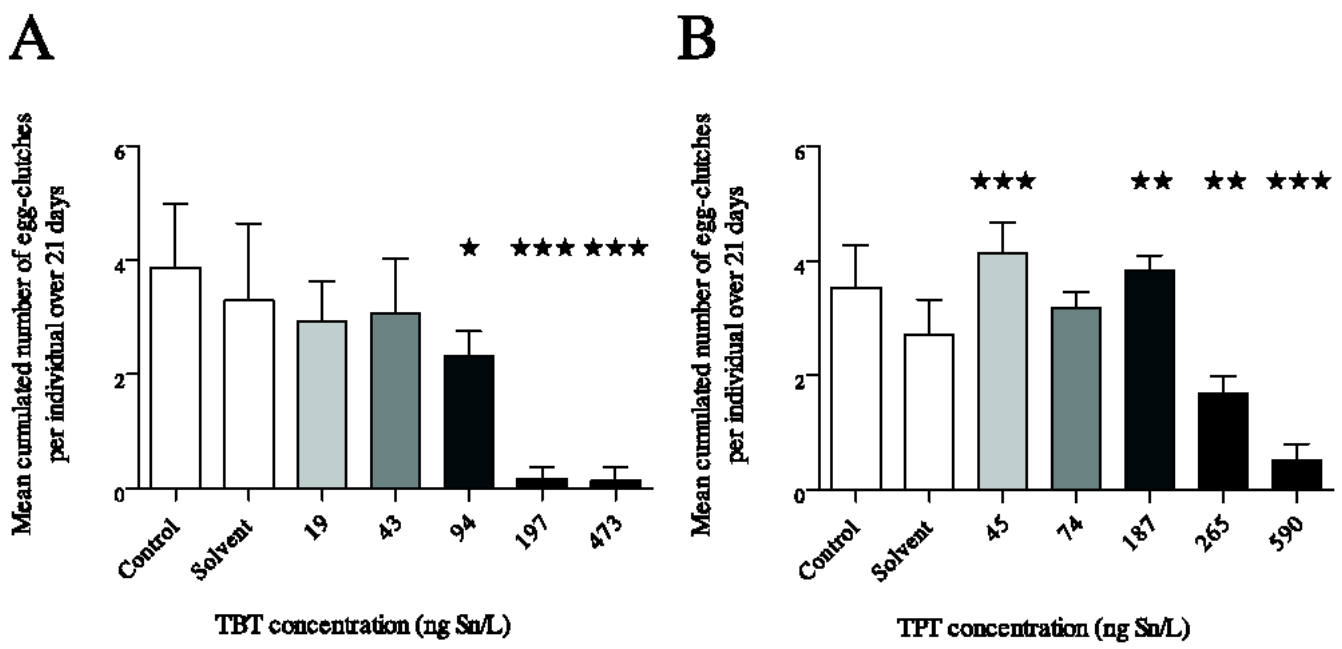

Figure 4

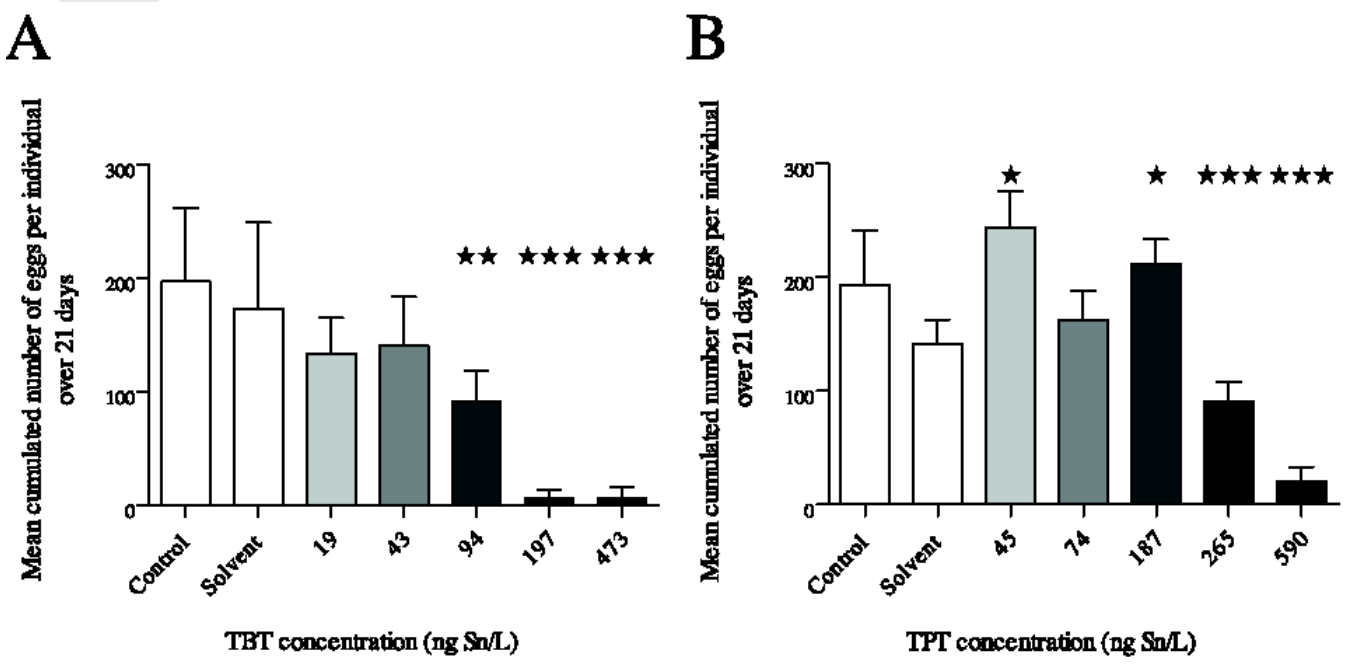

Figure 5

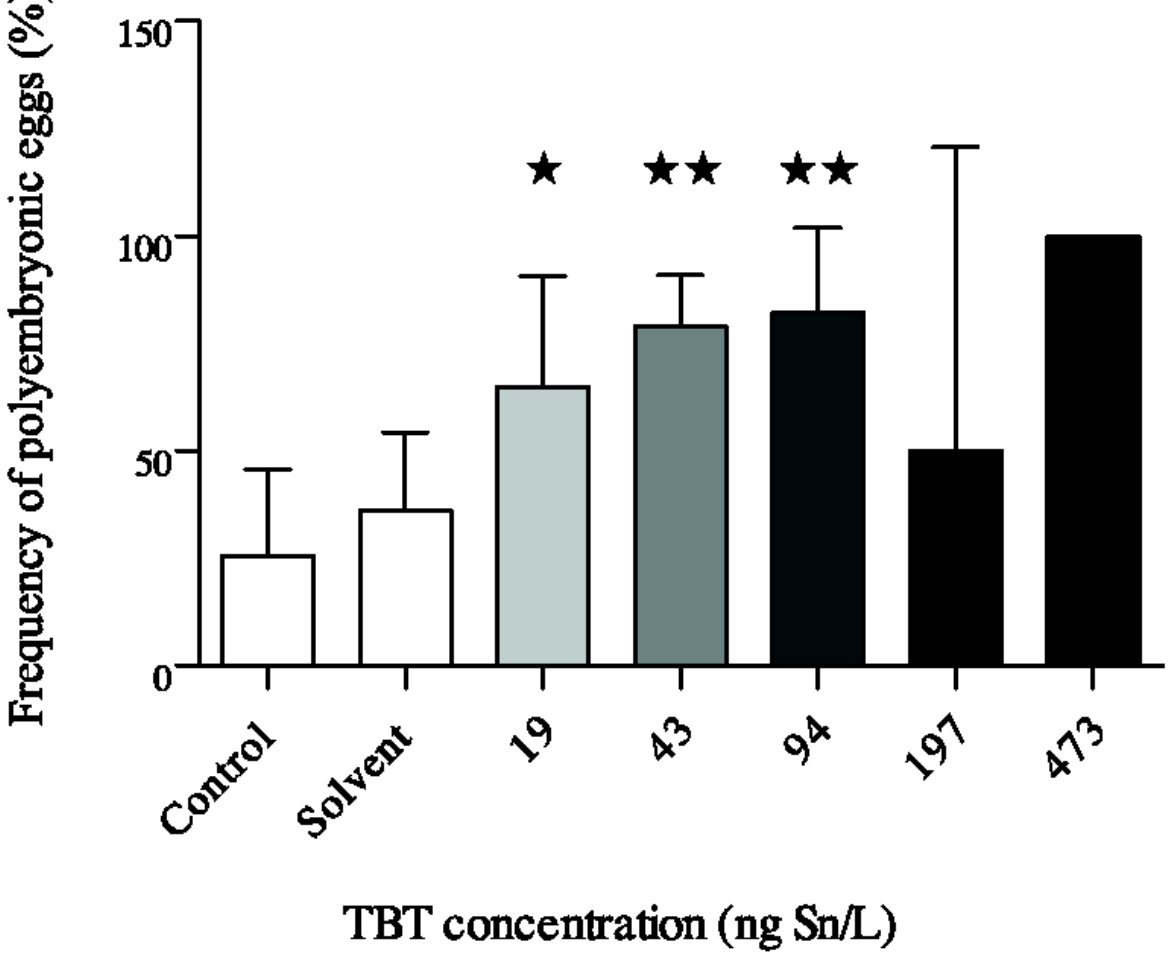

Figure 6