

REPRODUCTIVE IMPACTS OF TRIBUTYLTIN (TBT) AND TRIPHENYLTIN (TPT) IN THE HERMAPHRODITIC FRESHWATER GASTROPOD *LYMNAEA STAGNALIS*ARNAUD GIUSTI,<sup>†,‡</sup> ALPAR BARSÍ,<sup>‡</sup> MAËL DUGUÉ,<sup>‡</sup> MARC COLLINET,<sup>‡</sup> JEAN-PIERRE THOMÉ,<sup>†</sup> CÉLIA JOAQUIM-JUSTO,<sup>†</sup> BENOIT ROIG,<sup>§</sup> || LAURENT LAGADIC,<sup>‡</sup> and VIRGINIE DUCROT\*<sup>‡</sup><sup>†</sup>Laboratory of Animal Ecology and Ecotoxicology, Center of Analytical Research and Technology, Liège University, Liège, Belgium<sup>‡</sup>INRA, Joined Research Unit 985 Ecology and Ecosystem Health, Rennes, France<sup>§</sup>EHESP, Rennes, France

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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 to compare the relative toxicity of TBT and TPT under similar laboratory conditions in the range of 0 ng Sn/L to 600 ng Sn/L. 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 21-d semistatic test. Frequency of abnormal eggs was assessed as an additional endpoint. Triphenyltin 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, because snail responses to TBT could be detected at concentrations as low as 19 ng Sn/L. However, the putative mode of action of the 2 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 with the sensitivity of prosobranch mollusks with different habitats and different reproductive strategies. *Environ Toxicol Chem* 2013;32:1552–1560. © 2013 SETAC

**Keywords:** Reproductive toxicity    Endocrine disruptors    Mollusk toxicology    Organotin    Toxicity mechanisms

## INTRODUCTION

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

As early as 1975, adverse impacts of TBT—shell calcification in adults leading to stunted growth—were observed in the oyster

*Crassostrea gigas* [11]. At low concentrations (nanograms per liter), 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 mesogastropod and neogastropod marine and freshwater species [13]. At highly contaminated sites, females can be sterilized or even killed, which may affect population dynamics [14]. In addition, TPT was 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].

Organotins generally occur in the environment as mixtures of TBT and TPT and their derivatives (i.e., dibutyltin [DBT], monobutyltin [MBT], diphenyltin [DPT], and monophenyltin [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 toward the study of mixture toxicity. Comparisons of bioaccumulation and biological responses to TBT and TPT have already been done in several gastropod species such as the muricids *Thais clavigera* and *Thais bronni* [27], as well as *Bolinus brandaris* and *Hexaplex trunculus* [28]. These studies allow a straightforward comparison of TBT and TPT effects under similar test designs and experimental conditions and using similar test endpoints. This ensures both quantitative and qualitative reliability of effect comparisons through the avoidance of experimental confounding factors, which often occur when data from different studies are compared. To date, these comparative studies have dealt

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only with gonochoric species, in which 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 that exhibit separated genders, in which 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], the effects of organotins in these animals have not yet been extensively investigated [20]. In particular, it is important to investigate suitable endpoints that will allow the highlighting of reproductive effects in species in which sex differentiation cannot be used as an effect criterion. To date, most studies have been conducted using the great pond snail *L. stagnalis*. 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 mollusk species for assessing reprotoxic effects of chemicals [20,29,31–33]. Because its neurohormonal control of reproduction is reasonably well understood compared with other mollusk species [32–35], and also because it has been shown to be sensitive to endocrine disruptors [19,21,24,31,35], standard Organization for Economic Cooperation and Development (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 to investigate the reproductive effects of the emblematic organotin compounds TBT and TPT, known as endocrine disruptors in some mollusk species, in the hermaphroditic freshwater snail *L. stagnalis*. The 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 also were monitored. Effects of TBT and TPT were compared both qualitatively and quantitatively. The 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 with available data in other mollusk species.

## MATERIALS AND METHODS

### Test organisms

*Lymnaea stagnalis* (Linnaeus, 1758; Mollusca, Gastropoda, Panpulmonata, Heterobranchia) were reared at the Experimental Unit of Aquatic Ecology and Ecotoxicology (National Institute for Agricultural Research, Rennes, France) under laboratory conditions as previously described [30]. The culture medium consisted of dechlorinated, charcoal-filtered tap water with the following physicochemical characteristics: pH  $7.7 \pm 0.2$ , conductivity  $623 \pm 60 \mu\text{S}/\text{cm}$ , dissolved oxygen  $7.3 \pm 2 \text{ mg}/\text{L}$ , and water hardness  $254 \pm 7 \text{ mg CaCO}_3/\text{L}$ . Rearing conditions were as follows: temperature  $20 \pm 1^\circ\text{C}$ , photoperiod 14:10-h light:dark, and light intensity  $155 \pm 35 \text{ lux}$ . Snails (RENILYS® strain) were fed 3 times per week with organic lettuce. Young adult snails—in which reproduction endpoints are more sensitive to chemicals, including some endocrine-disrupting chemicals, than in fully grown snails [35]—were used. Snails of homogenous size ( $22.5 \pm 2.5 \text{ mm}$ ) and age

( $4 \pm 0.5$  months) were sampled from the culture and acclimated to test conditions (i.e., similar to culture conditions, but with a higher food quantity provided per individual) during the 48 h prior to chemical exposure.

### Tested chemicals and concentrations

Tributyltin hydride (Chemical Abstract Service [CAS] number 688-73-3) and triphenyltin chloride (CAS number 639-58-7) were dissolved in analytical grade acetone (99.9% purity) to prepare stock solutions ( $10 \mu\text{g}/\mu\text{L}$ ). Final solvent concentration ( $100 \mu\text{L}/\text{L}$ ) was homogenous among treatments, as recommended by the OECD [36] (except for the water controls). Exposure media consisted of culture water contaminated with stock solutions. Organotin concentrations were chosen based on 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 on this information, the chosen nominal concentrations were 45 ng Sn/L, 100 ng Sn/L, 220 ng Sn/L, 480 ng Sn/L, and 1065 ng Sn/L for TBT, and 100 ng Sn/L, 215 ng Sn/L, 755 ng Sn/L, 1000 ng Sn/L, and 2626 ng Sn/L for TPT. To facilitate the quantitative comparison of TBT versus 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 5 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 d because this duration was sufficient to provide evidence of effects and assess various effective concentration (EC<sub>x</sub>) values for the compounds tested in the proposed experimental conditions (as determined in pre-experiments). Test water was renewed with freshly contaminated medium to maintain exposure concentrations and adequate physicochemical properties of test water. Renewal rates resulted from a compromise between maintaining exposure concentration and avoiding too much stress to the snails. Independent studies showed that in our test conditions, TBT water concentration dropped rapidly (92% losses in 72 h), so that the TBT test medium was renewed every other day. The TPT was more stable (62% losses in 72 h), and thus the TPT test medium was renewed every 3 d. Tests were conducted at  $20.5 \pm 0.6^\circ\text{C}$  and in a 14:10-h 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 snail growth and reproduction [40].

Dead snails were counted and removed daily. Individual shell length was measured using a digital calliper at days 0 and 21 to assess a possible impact on growth. Effects on reproduction were estimated by monitoring the cumulated number of egg clutches per snail and the cumulated number of eggs per snail. Every day, clutches were counted, removed using a sharp-edged spoon, and photographed. The number and quality of the eggs were determined by observation of the photographs. Egg quality was assessed by determining the frequency of 4 types of abnormalities (Figure 1A–E): polyembryonic egg (the presence of several embryos per egg; unfertilized egg (the absence of embryo in the egg, which only consists of the eggshell and albumen; atrophied albumen (damaged eggshell containing an abnormally low albumen quantity); and single embryo (presence of a non-developing embryo, without an eggshell and without albumen). Polyembryonic and unfertilized eggs have been described before [41–43], and polyembryony has been shown to be a

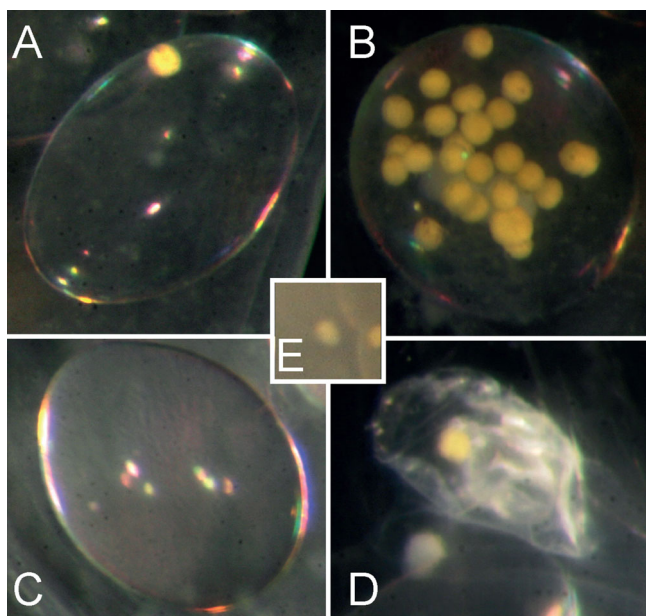


Figure 1. Different egg abnormalities observed in *Lymnaea stagnalis*. (A) normal egg; (B) polyembryonic egg; (C) unfertilized egg; (D) egg with atrophied albumen; and (E) single embryonic cell. [Color figure can be seen in the online version of this article, available at [wileyonlinelibrary.com](http://www.interscience.wiley.com)]

sensitive endpoint [41]. The 2 other abnormalities are described for the first time in *L. stagnalis*.

#### Chemical analysis

Water was sampled in controls and every tested concentration at the beginning, middle, and end of the tests. Water was collected both 15 min after stirring stock solutions in clean water (i.e., new exposure water) and just before water renewal (i.e., old exposure water) to allow calculation of time-weighted average exposure concentrations. For each concentration and sampling date, 3 samples of 1 L were collected, which consisted of a mixture of 165 mL of water extracted from each exposure replicate. In the TBT experiment, mucus that had accumulated on the walls of the test beakers was also collected for chemical analysis. Samples were frozen until analysis of their MBT, DBT, and TBT and TPT content. Analyses were performed by coupled capillary gas chromatography/mass spectrometry, 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 Belgers et al. [45]. Biological data were analyzed with standard statistical analysis procedures, as described by the OECD [46], using Sigma-Stat (Jandel Scientific) and GraphPad Prism 5.0 software. Analyses of survival and reproduction data were based on observations for the 6 replicates, and analysis of growth data was based on individual length measurements. Analysis of abnormality frequency was based on the total number of clutches collected 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 EC<sub>x</sub> values allows one to avoid 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 and size and Dunnett'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, lethal concentrations (LC<sub>x</sub>) or EC<sub>x</sub> were calculated using a logistic regression model [47]. The 95% confidence intervals (CI) were simulated based on weighted residues 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

The chemical preparation and contamination method resulted in exposure concentrations corresponding to  $62.7 \pm 14.2\%$  and  $87.5 \pm 17.6\%$  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  $35.7 \pm 4\%$  and  $18.1 \pm 3\%$  of the nominal TBT and TPT concentrations, respectively. This resulted in time-weighted average concentration ranges of 19 ng Sn/L, 43 ng Sn/L, 94 ng Sn/L, 197 ng Sn/L, and 473 ng Sn/L for TBT, and 45 ng Sn/L, 74 ng Sn/L, 187 ng Sn/L, 265 ng Sn/L, and 590 ng Sn/L for TPT. High TBT concentrations were also found in the mucus sampled after 21 d (e.g.,  $1563 \pm 638$  ng Sn/L at the highest tested concentration). Both MBT and DBT were found in water, due to the degradation of TBT. Concentrations of MBT and DBT were not included in the calculation of TBT time-weighted average concentrations because their concentrations were generally lower than the quantification limit, so they could be ignored.

#### 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 3 d of exposure ( $p < 0.05$ , Dunn's post hoc test), while lower concentrations did not significantly affect survival. The corresponding median lethal concentration (LC<sub>50</sub>) at 21 d was estimated to be 436.1 ng Sn/L (CI: 308.1–433.6 ng Sn/L).

#### Shell size and integrity

Shell length at 21 d was significantly reduced in snails exposed to TBT concentrations exceeding 94 ng Sn/L (Figure 2A). A nonsignificant 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 or all of the exposed individuals, leading to reduced shell length. Snails exposed to 0 ng Sn/L and 19 ng Sn/L generally did not suffer such injury. This finding suggests that broken shells were not experimental artifacts 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 (Figure 3A). Therefore, the reduction in shell length provides evidence for the effects of TBT on shell solidity. Analysis of size data only in snails that did not suffer shell injury highlighted a significant reduction in growth

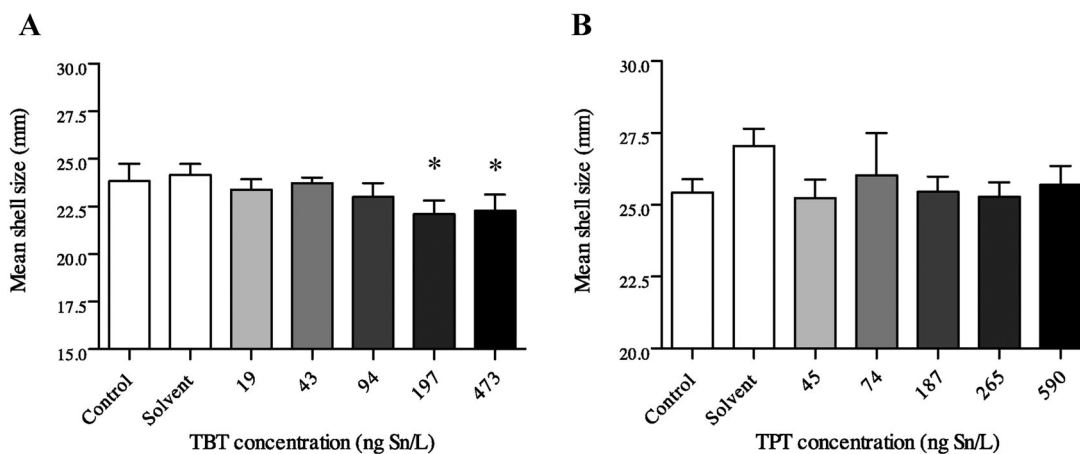


Figure 2. Mean shell size after a 21-d exposure to (A) tributyltin (TBT) or (B) triphenyltin (TPT). Error bars represent standard deviation over 6 replicates (\*  $p < 0.05$ ).

for TBT concentrations exceeding 94 ng Sn/L. As the growth effect was not very intense in the concentration range tested, no reliable  $EC_x$  value could be calculated. Damaged shells were observed at all TPT concentrations tested (but not in controls), suggesting that TPT also had an impact on shell solidity (Figure 3B). Damage frequency was between 50% and 70%, regardless of exposure concentration. No significant effect on growth was found with TPT (Figure 2B).

#### Egg-laying behavior

The time-course of effect was different in snails exposed to TBT concentrations of 94 ng Sn/L or less: egg-laying occurred regularly but at a slower rate than in controls. For snails exposed to 197 ng Sn/L and 473 ng Sn/L, egg-laying ceased after 1 wk of exposure. The cumulated number of clutches produced per individual over 21 d decreased in all snails exposed to TBT compared with the water controls, from -24% at 19 ng Sn/L to -96% at 473 ng Sn/L (Figure 4A). A significant difference from solvent controls in the number of clutches produced ( $p < 0.05$ , Dunnett's post hoc test) was detected at 94 ng Sn/L. Exposure to the 2 highest TBT concentrations resulted in a severe reduction in egg-laying activity ( $p < 0.001$ , Dunnett's post hoc test). The corresponding median effective concentration ( $EC_{50}$ ) at 21 d was estimated to be 118.3 ng Sn/L (CI: 99.7–171.9 ng Sn/L).

In the TPT test, the time-course of effect was similar in snails exposed to all concentrations tested; 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 (Figure 4B). Solvent control was thus used as a reference for statistical tests for both TPT and TBT. Exposure to 265 ng Sn/L and 590 ng Sn/L caused a significant decrease ( $p < 0.01$  and  $p < 0.001$ , respectively, Dunnett's post hoc test) in the cumulated number of clutches produced (-38% and -81% of the solvent control value, respectively). Based on these results, the estimated  $EC_{50}$  21-d value was 264.1 ng Sn/L (CI: 258.5–280.5 ng Sn/L).

#### Fecundity

The effect patterns and estimated no-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) values of TBT and TPT for fecundity were similar to the patterns observed for egg-laying behavior: the LOEC values were 94 ng Sn/L and 264 ng Sn/L ( $p < 0.01$  and  $p < 0.001$ , respectively, Dunnett's post hoc test) for TBT and TPT, respectively.

Compared with water controls, a nonsignificant reduction in fecundity was already observed at the 2 lowest TBT concentrations and was particularly severe at the 2 highest tested concentrations (from -32% at 19 ng Sn/L to -97% at 473 ng Sn/L,

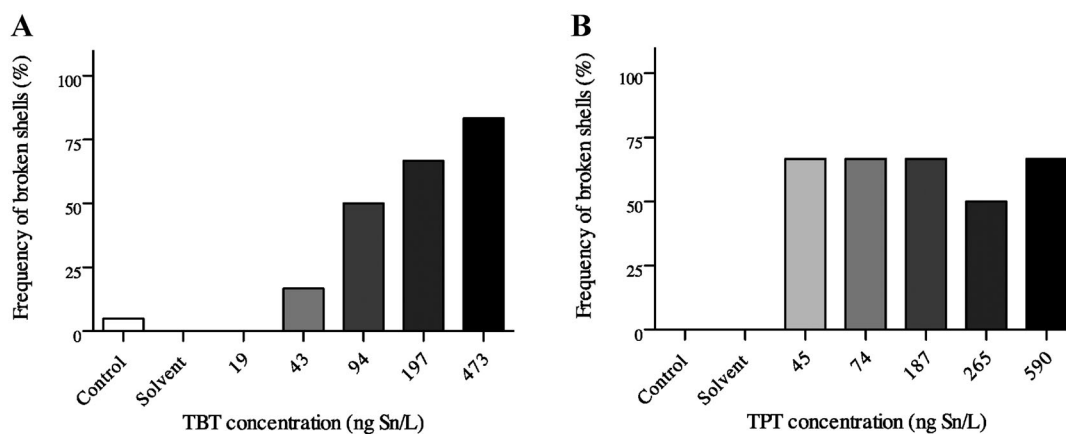


Figure 3. Frequency of broken shells observed over 6 replicates after a 21-d exposure to (A) tributyltin (TBT) or (B) triphenyltin (TPT).

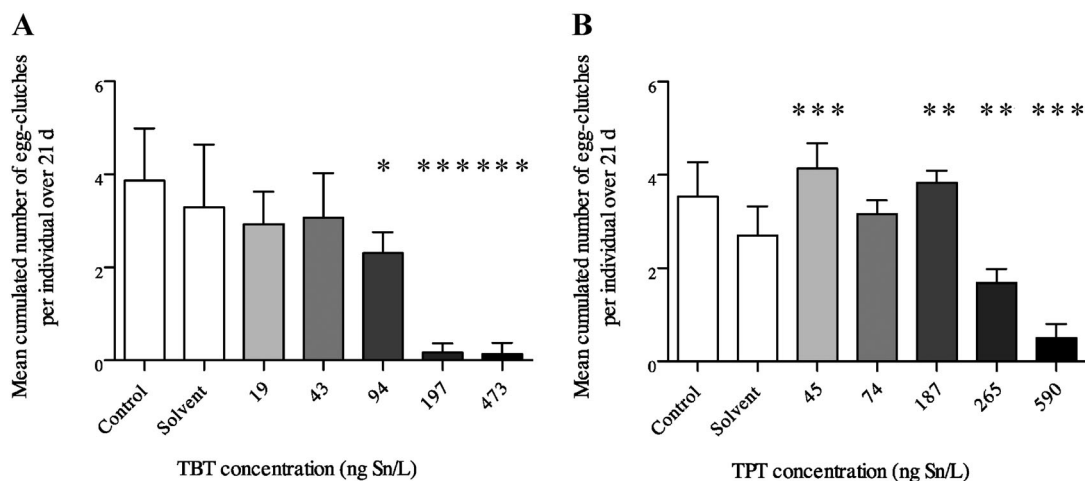


Figure 4. Mean cumulated number of egg-clutches laid per individual after a 21-d exposure to (A) tributyltin (TBT) or (B) triphenyltin (TPT). Error bars represent standard deviation over 6 replicates (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ).

as shown in Figure 5A). The corresponding EC<sub>50</sub> 21-d value was 106.2 ng Sn/L (CI: 84.4–125.6 ng Sn/L), which was not significantly different from the EC<sub>50</sub> at 21 d 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 2 highest TPT concentrations (265 ng Sn/L and 590 ng Sn/L) was significantly reduced (–36% and –85% of the solvent control value, respectively), as shown in Figure 5B. The corresponding EC<sub>50</sub> 21-d value was 263.9 ng Sn/L (CI: 253.8–280.5 ng Sn/L), which was similar to the EC<sub>50</sub> at 21 d found for oviposition.

#### Egg-abnormalities

Frequency of abnormal eggs per clutch increased over the test duration. After 21 d, 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). The 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, representing 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 compared with control (Figure 6). This increase was significant for all concentrations tested ( $p < 0.05$ , Dunnett's post hoc test). The corresponding EC<sub>50</sub> at 21 d was 23.7 ng Sn/L (CI: 2.2–189.4 ng Sn/L). All eggs produced by parents exposed to 473 ng Sn/L exhibited polyembryony. In contrast, 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*

The TBT metabolite concentrations were very low (i.e., below the quantification limit), which suggests that degradation might not be the most significant process contributing to the decrease in TBT concentration in water. Losses were probably mostly due to adsorption in mucus, for example. Indeed, high TBT concentrations—3- to 5-fold higher concentrations than the measured peak concentration in water—were found in mucus sampled after 21 d. The TBT content in mucus most likely resulted from direct adsorption from water after the mucus was

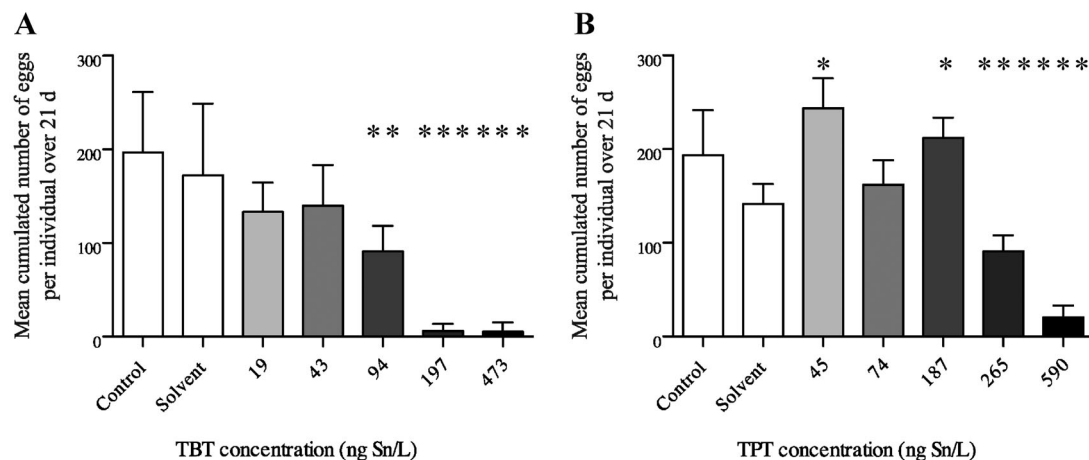


Figure 5. Mean cumulated number of eggs laid per individual after a 21-d exposure to (A) tributyltin (TBT) or (B) triphenyltin (TPT). Error bars represent standard deviation over 6 replicates (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ).

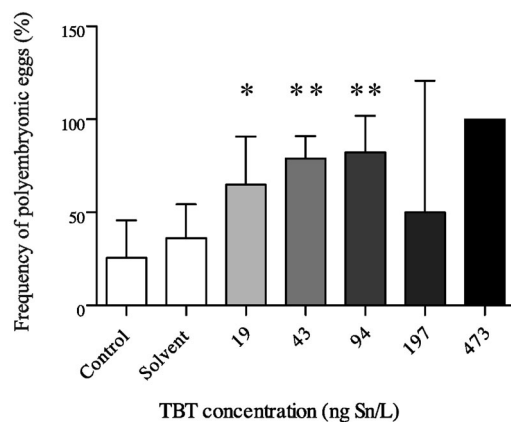


Figure 6. Frequency of the polyembryonic eggs (among the total number of abnormal eggs) found during a 21-d exposure to tributyltin (TBT). Error bars represent standard deviation over all abnormal eggs produced (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ).

released and possibly, to a lesser extent, from elimination of TBT by snails via mucus. This adsorption might partly explain why only 62.5% of the targeted nominal concentrations were found in the water samples. This highlights the need to wipe mucus off test beakers between water renewals to limit adsorption of TBT on the test chamber walls, which reduces its availability to the snails.

Exposure of *L. stagnalis* for 21 d to TPT in the concentration range of 45 ng Sn/L to 590 ng Sn/L induced mortality at the highest tested concentration (LC50 at 21 d, 436.1 ng Sn/L), and a variety of sublethal effects at lower concentrations, that is, decrease in shell solidity at all tested concentrations, decrease in egg-laying activity and fecundity, endpoints that led to an identical EC50 at 21-d value of 264 ng Sn/L. Egg quality at the concentration range tested was not affected by TPT.

A 21-d exposure of *L. stagnalis* to TBT in the range of 19 ng Sn/L to 473 ng Sn/L did not result in significant mortality in the present study. Alternatively, Segner et al. [24] showed that adult survival was significantly reduced by TBT exposure, with an LC50 at 21 d of 290 ng Sn/L (nominal concentration), which is in contrast to both the present results 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 56 d of exposure to the concentration range of 7 ng Sn/L to 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 in which snails were exposed to 410 ng Sn/L [19].

Exposure to TBT induced a decrease in growth at concentrations exceeding 94 ng Sn/L. It also induced a decrease in shell solidity in snails exposed to concentrations exceeding 43 ng Sn/L, which was also observed by Segner et al. [24] in 21-d tests for nominal concentrations exceeding 94 ng Sn/L. 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 in egg-laying at 100 ng Sn/L (nominal concentration) 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], in which animals were exposed from 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 the present study (NOEC, 43 ng Sn/L). However, the effect pattern was quite different (S-shape dose-response curve in the present study vs inverted U-shape in the study of Leung et al. [19], with an increase in fecundity at 41 ng Sn/L compared with controls). The magnitude of effects at 410 ng Sn/L was also different. Indeed, the fecundity was reduced by a factor of 10 compared with control in the study of Leung et al. [19], whereas it was reduced by a factor 100 in the present 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 to compare the sensitivity of partial versus full life-cycle tests 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 finding 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 greatly 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 versus TPT

Differences in the sensitivity of *L. stagnalis* to similar concentration ranges of TBT and TPT were observed for most of the endpoints studied. The TBT concentrations tested (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 TBT to *L. stagnalis*. Results from a pretest with TBT confirmed that TBT has no effect on survival at 590 ng Sn/L. (The NOEC in this pretest was 627 ng Sn/L, while the LOEC was 6270 ng Sn/L, with 100% mortality occurring at the end of the test; data not shown.) A significant effect on snail growth was found with TBT, 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 the effects of organotin on *L. stagnalis*. Indeed, decreased shell size due to an injury can be wrongly interpreted as an effect on growth. 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 loss of a number of data and thus to a reduced statistical power when effects on growth are tested (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 to avoid 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 mollusks (e.g., oysters [49]). Similarly, trialkylated 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 EC50 21-d values for reproductive endpoints. The EC50s at 21 d based on fecundity data were equivalent to values obtained from oviposition data for TBT (EC50 21-d values of 106 ng Sn/L 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 21-d 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 2 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 different responses to TBT and 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 significantly hamper all reproductive performances in the TPT test. Previous in-house studies showed that reproductive effects due to acetone occur from time to time in our experimental conditions. The reasons remain unclear; this may be a result of, for example, differences in solvent purity or snail sensitivity from one experiment to another. This highlights the need for additional studies devoted to the assessment of solvent effects in juveniles and adults of *L. stagnalis* to complement the recently published results on solvent effects in embryos [43].

Reasons for differences in sensitivity of *L. stagnalis* to TBT versus TPT remain to be elucidated. Differences in molecular structure of these organotins might be a relevant explanation. Indeed, it is assumed that the toxicity of organotins is more influenced by the alkyl substitutes than by the anionic substitutes [6]. A recent study in *Mytilus edulis* confirmed that alkylation of organotins influences their toxicity to mollusks [50]. Therefore, differences in the responses of *L. stagnalis* to TPT versus TBT are likely to be related to differences in their alkylation. However, it is not known how such differences in molecular structure 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 the fact that imposex is related to an alteration in steroid homeostasis, mediated by inhibition of enzymes such as cytochrome-P450 aromatase or acyltransferase that are involved in biosynthetic steroid 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, leading to transcription of genes involved in steroid homeostasis [52,60–63]. Other studies highlighted the effects of TBT and TPT on different components of the microsomal monooxygenase system of the bivalves *Mytilus galloprovincialis* and *Ruditapes decussata* and of the gastropod *Thais haemastoma* [64]. Finally, Lyssimachou et al. [15] showed that TPT was able to alter lipid metabolism in females of the ramshorn snail *Marisa cornuarietis*. However, it is not known to what extent these findings can be extrapolated to

hermaphroditic species such as *L. stagnalis*. In the present study, reproductive effects of TPT occurred at quite high concentrations (i.e., the LC50 at 21 d was only 2-fold higher than the reproductive EC50 at 21 d). Values for EC50 at 21 d 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 abnormalities occurred in the offspring of exposed snails. Therefore, results of the apical reproduction test suggest that the reproductive impacts observed 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* as well. Even if apical endpoints might bring clues to possible modes of action of TBT and TPT in *L. stagnalis*, studies should be implemented to determine 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 and gonochoric species*

Existing data, including the present results, have shown that TPT and TBT have different acute and chronic effects in various mollusk species. For instance, exposure of the parthenogenetic snail *Potamopyrgus antipodarum* to TBT and TPT in sediment biotests led to a significant reduction in 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 compared with those exposed to TBT. In contrast, acute toxicity occurred in snails exposed to TBT but not in those exposed to TPT. Based on these results, the effects of TPT and TBT in *P. antipodarum* are opposite to the effects we found in *L. stagnalis*. In another study, it was shown that females of the rock shell *T. clavigera* had a 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 3 prosobranch snails with different habitats (freshwater, brackish water, and marine water) and different reproductive strategies (parthenogenesis, hermaphroditism, and gonochorism) were all responsive to organotin compounds, but their sensitivities were species- and sex-dependent, as reviewed by Ketata et al. [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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