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Environmental Toxicity Assessment of Organotin Compounds Using the Direct Contact Bioassay – OSTRACODTOXKIT F

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Abstract
The aim of the study was to assess the toxicity of organic compound of tin using two tests with Heterocypris incongruens.
Chlorides: tributyltin, trimethyltins and triphenyltin were tested by conducted acute (mortality) and chronic toxicity tests (mortality and length of organisms).
The highest acute toxicity were obtained by TBT (LC50=0.113 ppm) and TPT (LC50=0.167 ppm), while the lowest was TMT (LC50=1.76 ppm).
In chronic lethal effect was caused by the same TPT concentration (above 0.1 ppm), whereas different results were obtained for TBT (mortality in concentrations over 10-times higher), which may be due to strong binding of compound with sediment

Keywords: Ostracodtoxkit, tributyltin, tin, Ostracod, toxicity

1.1.1 Introduction

Tin is a chemical element with the symbol Sn (Latin: Stannum). It belongs to group 14, period 5 of the periodic table, is part of the p-block, and its electron configuration is [Kr] 4d10 5s2 5p2. Under standard conditions, tin is a solid metal with 10 stable isotopes, typically forming compounds in the +2 (e.g., $Sn(OH)_2$) and +4 (e.g., $SnCl_4$) oxidation states.
In Poland, tin has historically been mined in areas such as Gierczyn, the Sudetes, and Czarnów [1, 47] , due to its broad industrial applications: as an anti-corrosion coating in cans, for alloy casting in metallurgy, in electronics for soldering, and in the aerospace industry—combined with titanium—to produce lightweight, high-strength materials [2].
In 1849, British chemist Edward Frankland, notable for introducing the concept of valency, synthesized the first organotin compound: diethyl diiodotin $(C_2H_5)_2SnI_2$ [3]. The organotin chemistry field rapidly expanded after 1900, driven by the discovery of the Grignard reagent, which enabled efficient formation of Sn–C bonds [7].
Since then, the number of known organotin compounds has grown substantially, with most featuring tin in the +4 oxidation state [5].

1.1.2 Organotin Compounds as Marine Antifouling Coatings

Since the earliest use of boats, maintaining hull integrity has been vital. A major concern today is marine biofouling—the colonization of ship hulls by aquatic organisms. These biofilms: decrease hydrodynamic efficiency and speed, increase total vessel mass, impair manoeuvrability, mechanically damage hull coatings (e.g., shipworm infestations).
These effects cumulatively lead to increased fuel consumption, longer transport times, higher maintenance costs, and reduced safety and operational efficiency [45].
The urgent need for effective antifouling solutions led to advancements during WWII, culminating in a comprehensive 1952 guide from the Woods Hole Oceanographic Institution

on antifouling coatings [44].
This spurred widespread industrial interest, leading to the development of halogenated organotin derivatives—especially tributyltin chloride (TBTCl) and triphenyltin chloride (TPTCl)—which exhibited potent antifouling efficacy [8].
Despite their industrial utility, their use has since been drastically reduced or banned due to high toxicity to marine life [17, 45], extreme lipophilicity [35], and long-term environmental persistence (up to 30 years) [18].
In the European Union, organotin antifouling agents were banned under Directive 76/769/EEC and Regulation (EC) No 782/2003, as well as Directive 98/8/EC on biocidal products [34]. However, countries with poor regulatory oversight (e.g., Nicaragua, Costa Rica, Trinidad and Tobago) may still use these compounds.
Current antifouling solutions are primarily copper oxide- or zinc-based and are classified as: ablative (soft): Time-dependent release of active compounds facilitated by water movement; common in small vessels. Requires reapplication every 1–3 years [45], hard coatings: Include biocidal pore-forming films, slippery Teflon/silicone surfaces, and specialized systems like SealCoat Systems® with micro-fibers that prevent organism adhesion.

1.2.1 Tributyltin Chloride (TBTCl)

Tributyltin chloride [$(C_4H_9)_3SnCl$] is a colourless liquid produced via redistribution of tetrabutyltin with tin(IV) chloride under controlled conditions [15].
It is used in PVC stabilization, antifungal applications, and primarily as an antifouling agent since the 1960s [9, 26].
Numerous studies confirm its severe toxicity to aquatic invertebrates and vertebrates [13, 29, 31, 32, 43]. Even at nanomolar concentrations: arthropods: induction of irreversible masculinization in females via RXR receptor activation at 1 ng/L [39], fish: altered sex ratios, fertility suppression, and hepatic signalling disruption [27, 37], mammals: activation of RXR and PPARγ receptors, leading to hepatic steatosis and metabolic dysfunction [42, 26, 28]
Despite the ban, TBT is still detected. A 2013 study in Gdynia Port found concentrations of 200–400 ng/L in water and 1–10 μg/L in sediment [36].

1.2.2 Trimethyltin Chloride (TMTCl)

Trimethyltin chloride [$(CH_3)_3SnCl$] is a solid, malodorous compound produced via redistribution reactions involving tin(IV) chloride or through acid-base reactions with trimethyltin hydroxide [38].
Used industrially for $CH_3SnCH{=}CH_2$ monomer production, PVC stabilization, and as a pesticide [5, 19], TMT is released into soil and water by industrial waste.
TMT causes severe neurotoxicity in humans, notably limbic-cerebellar syndrome, with symptoms such as memory loss, confusion, seizures, and even death [11, 41].
Animal models show hippocampal apoptosis, astrocyte activation, and gliosis [21].
In *Danio rerio* embryos, exposure led to increased mortality, deformities, edema, reduced body length, and dysregulated neurodevelopmental gene expression [23, 12].

1.2.3 Triphenyltin Chloride (TPTCl)

Triphenyltin chloride is a solid organotin compound used historically as a fungicide, pesticide, and antifouling agent. Although banned in coatings, it remains in agricultural use—particularly in Asia [34].
TPT toxicity is similar to potassium cyanide [14], acting through RXR receptor activation [20].
In rodent studies, doses of 6 mg/kg induced high mortality, while lower doses (2 mg/kg) caused hormonal disruption, genital deformities, and organ enlargement [16].
In *Danio rerio*, TPT exposure caused eye malformations, including retinal and lens damage [46].

1.3 Assessment of Toxic Effects

The primary objectives of toxicological studies on samples previously collected from the environment are: risk assessment and determination of the dose causing a toxic effect.

Risk assessment aims to estimate the likelihood of a toxic effect occurring, while the toxic dose defines a quantitative threshold that induces a toxic response.

Another important aim of such studies is the evaluation of long-term effects of exposure to toxic agents, including the classification of effects into categories such as mutagenic, carcinogenic, etc. [25].

These studies can be divided into: toxicological field studies, involving observation of biota under exposure to environmental agents, Laboratory toxicological studies, using advanced analytical techniques [25].

1.3.1 Instrumental Methods

Among the currently used instrumental analytical techniques, the following can be distinguished: high-performance liquid chromatography (HPLC), mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR).

The main advantage of these methods is their high detection sensitivity, enabling both quantitative and qualitative analyses at concentrations as low as ppb (µg/l) or even ppt levels [24].

While MS and NMR are primarily used for qualitative analysis, HPLC is employed for both qualitative and quantitative purposes [30].

Nowadays, hybrid techniques are increasingly used for greater precision and efficiency, such as LC-MS and GC-MS [24].

1.3.2 Biological Methods

Biomonitoring refers to the analytical examination of living biological material or tests based on biological indicators, using plant or animal organisms to respond to toxic agents.

Another category of biological methods is bioanalytic, which relies on biotests using biological samples as control and measurement materials, or biosensors, which are integrated with analytical devices [25].

1.3.3 Bioindication and Monitoring Methods

Bioindication methods use living organisms as indicators, allowing non-invasive assessment of ecological changes.

These changes reflect overall toxicity, rather than exact concentration or compound identification as in instrumental methods [48].

Examples include: the presence or absence of lichens (Lichenes) as an indicator of sulfur dioxide air pollution [48], fish, which are widely used as bioindicators — in Europe, 60 out of 122 species used in toxicity testing are fish, invertebrates, such as crustaceans, rotifers, and Daphnia, algae, which are notable for allowing both toxicity and growth stimulation assessment [10].

Currently, standardized bioindication test kits with reagents are being developed, enabling mobile, simple, and reproducible testing with sensitivity sometimes comparable to instrumental methods.

1.4 OSTRACODTOXKIT F™ Test [33]

The Ostracodtoxkit F™ is a modern bioindication test categorized as a "direct contact" test, as it assesses toxicity of both dissolved and undissolved substances that often accumulate in sediments or soil [OSTRACODTOXKIT F™].

The test organism is the ostracod Heterocypris incongruens, a small crustacean found worldwide in both marine and freshwater habitats.

They feed mainly on cyanobacteria, algae, protozoa, and decaying organic matter.

Their natural habitat is the benthic zone, where they swim and burrow in sediments.
In aquatic ecosystems, they act as saprophages, accelerating organic matter decomposition, and they also serve as a food source for fish and birds [22, 40].
The Ostracodtoxkit F™ kit contains all necessary components for sample analysis. The primary test endpoint is mortality, with growth inhibition as an optional secondary endpoint.
It is intended for evaluating the toxicity of sediments and soils [OSTRACODTOXKIT F™].
This test was selected for the assessment of organic tin compounds, considering their ability to adsorb to bottom sediments. An additional advantage is its high sensitivity and accuracy [OSTRACODTOXKIT F™].

2. Aim of the study

The aim of this study is to assess the toxicity of organotin compounds using acute and chronic toxicity tests on the ostracod *Heterocypris incongruens*.
The experiment will involve a 24-hour acute toxicity test and a 6-day Ostracodtoxkit F™ test.
In the acute toxicity test, the endpoint will be mortality of the organisms, while in the Ostracodtoxkit F™ test, in addition to mortality, organism length will also be evaluated.
All materials and biological components required for both tests are standardized, ensuring high sensitivity and reproducibility of the results.
Among the selected organotin compounds, the study will focus on the following chlorides: tributyltin chloride, trimethyltin chloride, and triphenyltin chloride.
Based on the results obtained, the sensitivity of the test organisms to the selected organotin derivatives will be analysed and compared with toxicity data from studies using other test organisms.

3. Material and methods

The compounds selected for this study are organotin derivatives, namely:
1) Tributyltin chloride - TBT
2) Trimethyltin chloride - TMT
3) Triphenyltin chloride – TPT

3.2 Bioindication Test – *OSTRACODTOXKIT F™*

3.2.1 Test Organism – *Heterocypris incongruens*

These small organisms, belonging to the class Ostracoda (ostracods), have a body enclosed in a bivalve-like shell consisting of two parts. Their external anatomy includes seven pairs of appendages—four on the head and three on the thorax.
Ostracods are dioecious, with separate male and female individuals, although sexual dimorphism is only weakly marked. Their diet consists of algae, cyanobacteria, protozoa, and plant and animal detritus [40, 22].

3.2.2 Test Characteristics

The Ostracodtoxkit F™ is also known as a chronic toxicity test for crustaceans or a "direct contact test." It was developed by the Laboratory of Environmental Toxicology and Aquatic Ecology under the leadership of Prof. G. Persoone at Ghent University, Belgium.
The test is designed to determine the percentage mortality and growth (length) of *Heterocypris incongruens* hatched from cysts after six days of exposure. The results are compared to a negative control sample- a non-toxic sediment.
A key advantage of the test is its mobility, as the organisms are provided in the form of dormant cysts that can be stored and activated on demand, just prior to testing.
Another benefit is the global distribution of *H. incongruens*, which occurs naturally in freshwater bodies around the world.
The high accuracy of the test is ensured by the reproducibility of the standardized materials provided in the kit.
The sensitivity of the Ostracodtoxkit F™ chronic sediment toxicity test has been shown to be

comparable to or even greater than that of the 10-day Hyalella azteca sediment toxicity test, and significantly higher than tests using Chironomus riparius larvae [4].

3.2.3 Test Kit Components

The TOXKIT is a microbiotest that contains all components necessary for conducting the assay, including the test organisms. It is characterized by its simplicity, efficiency, and reliability. All components are single-use, and the synthetic materials are non-toxic.

Required equipment includes a thermostated incubator set at 25°C, a microscope with a micrometric scale, and a digital camera connected to a computer.

Ostracod Cysts

Six 1 cm^3 vials containing standardized amounts of *H. incongruens* cysts. To ensure maximum hatching potential, they should be stored at 5°C ± 2°C. Proper storage and hatching procedures ensure a sufficient number of organisms for testing.

Multi-well Test Plates

Polystyrene 6- and 24-well plates used as test chambers.

Polyester Sealing Strips

Adhesive strips used to seal the wells, minimizing evaporation and preventing cross-contamination of vapor phases during incubation.

Petri Dishes

Six polystyrene Petri dishes (5 cm diameter) with lids: three for hatching, and three for transferring and counting organisms at the end of the test.

Concentrated Salt Solutions

Five bottles of concentrated salts for preparing 1 liter of EPA Standard Medium, used for cyst hatching, algal suspensions, and during the assay.

Control Sediment with Spatulas

The negative control consists of non-toxic quartz sand. In this experiment, lyophilized sediments from the Szczecin Lagoon were used, due to their potential to adsorb organotin compounds. Sediments are stored in plastic containers with a 500 µL measuring spoon.

Spirulina Powder

Six tubes of powdered *Spirulina* for feeding the young ostracods.

Micropipettes

A set of six standard glass micropipettes for transferring organisms, and six wide-tipped micropipettes for handling organisms during post-incubation processing.

Lugol's Solution and Thiosulfate

A small bottle of Lugol's iodine solution is used to euthanize organisms for length measurements. A thiosulfate solution is included to neutralize excess iodine.

Micrometric Calibration Slide

A transparent 2×2 cm calibration slide with a 1 cm micrometric scale (50 µm units) used to calibrate organism length measurements from digital images.

Micro sieves

Two sieves with 100 µm mesh for removing fine sediment particles after the test.

User Documentation

A detailed manual and quick-start guide providing step-by-step instructions for conducting the Ostracodtoxkit F™ test.

Score Sheet

Used for recording organism mortality and growth data.

Specification Sheet

Includes expiration dates for the cysts, batch numbers for salts, and medium composition details.

3.2.4 Equipment and Materials Required for the Test

MILLIPORE water purification system (for deionized water), Incubator and thermostated chamber (set to 25ºC), MPW-6 centrifuge, SONUPULS GM 200 BANDELIN ultrasonic homogenizer, SHIMADZU UV-1201 UV-VIS spectrophotometer, NIKON SMZ1500 microscope with camera, Computer equipped with Microsoft Office Excel™ and ImageJ™ software, Set of automatic pipettes (volume range: 50 µL to 10 mL).

Glassware: 10 mL test tubes, Petri dishes, Erlenmeyer flasks, Beakers (50–500 mL).

Other Materials: test tube rack, 50 mL eppendorf-type tubes, polystyrene microplates (6-well format), disposable pipette tips.

3.2.5 Methodology

3.2.5.1 Preparation of EPA Standard Medium

The EPA standard medium was prepared according to the test manufacturer's guidelines. Four concentrated salt solutions ($NaHCO_3$, $CaSO_4$, $MgSO_4$, KCl) were combined in a 1-liter volumetric flask. Deionized water was added to the mark, and the mixture was vigorously shaken to aerate the solution.

The resulting medium should have a pH of 7.6 ± 0.2. If necessary, the pH was adjusted using 1 M NaOH or 1 M HCl.

The prepared EPA medium was stored in a cool and dark location, and was thermostated to room temperature before each use.

This medium was used for cyst hatching, organism maintenance, and as a solvent for both the Ostracodtoxkit F™ and acute toxicity tests.

3.2.5.2 Hatching of Test Organisms

The recommended hatching time for *Heterocypris incongruens* is approximately 52 hours before the start of the experiment.

To initiate hatching, 8 mL of prepared EPA medium was added to a Petri dish. The vial containing cysts was emptied into the dish and rinsed twice with 1 mL of EPA solution each time to ensure complete transfer. Each rinse was also added to the dish.

The Petri dish was covered with a lid and placed in an incubator set at 25°C with illumination of 3000–4000 lux, and incubated for 52 hours.

3.2.5.3 Preparation of Algal Suspension (Food Source)

Algae used in the test were cultured in the Environmental Research Department.

Algal cultures were transferred to test tubes and centrifuged for 10 minutes at 3000 rpm. The supernatant was discarded, and the resulting pellet was transferred to a flask and resuspended in 35 mL of EPA medium.

The solution was vigorously shaken, then diluted tenfold. Absorbance was measured at λ = 678 nm, and the suspension was used if it showed an absorbance in the range of A = 0.3–0.36, corresponding to an initial optical density of A = 3.0–3.6.

3.2.5.4 Preparation of Tested Solutions

For each tested compound (TBT, TMT, TFT), a stock solution (referred to as [SS]) was prepared using pure compounds obtained from Sigma Aldrich®. Each [SS] was prepared by dissolving 10 mg of the compound in 10 mL of solvent, resulting in a concentration of 1 mg/mL (i.e., 1000 ppm).

Each [SS] was then diluted tenfold to create working solutions [WS] with concentrations of 0.1 mg/mL (100 ppm).

For TBT and TMT, methanol (HPLC grade) was used as the solvent for both [SS] and [WS].

For TPT, dimethyl sulfoxide (DMSO, HPLC grade) was used to prepare the [SS], and EPA medium was used as the diluent for [WS].

3.2.5.5 Study of the Effect of Oxygen Conditions on Ostracod Length

The purpose of this experiment was to determine whether the absence of air in the test chambers affects the survival and body length of the test organisms.

Two 6-well plates were prepared: one designated as the aerobic culture and the other as the anaerobic culture.

Each well was filled with: 3 mL EPA medium, 1 mL freshly prepared algal suspension, 10 juvenile ostracods, Two spatulas (~1 mL) of sediment from the Szczecin Lagoon

The plate labeled as anaerobic was additionally sealed with adhesive tape to minimize gas exchange. Both plates were covered and placed in an incubator at 25°C for six days.

At the end of the incubation, the length of the surviving organisms was measured using a camera-mounted microscope connected to a computer.

Organism lengths were analysed using ImageJ™ software.

3.2.5.6 Preliminary Chronic Toxicity Test of TBT, TMT, TPT

A preliminary chronic toxicity test was conducted for each of the tested compounds to select the appropriate concentration range for the final test.

For each compound, two replicates were prepared for each of three concentrations, along with three replicates as negative, non-toxic control trials. In total, 21 test wells were prepared.

To obtain the desired dilutions of the substances, working solutions [RR] were further diluted with standard EPA medium using a five-fold serial dilution method.

Filling of Test Vessels

Each test vessel of every subsequent plate, well, and replicate was filled following the standard routine procedure: 1 ml of EPA medium, 1 ml of freshly prepared algae (A = 3.0–3.5, at λ = 678 nm), Using a glass micropipette, 10 ostracods were transferred, 2 spatulas (total 1 ml) of lyophilized sediment, 2 ml of the test substance at double the target concentration (i.e. 2x concentrated for final dilution) (TBT/TMT/TPT), or 2 ml of EPA for control trials.

The prepared test plates were placed in a thermostatically controlled incubator at 25°C for six days.

Mortality

After six days of incubation, live and dead organisms were counted under a microscope for each well. Organisms capable of movement were considered alive, while immobile or missing individuals were classified as dead.

To simplify counting, the contents of each well were transferred onto a 100 µm sieve and thoroughly rinsed with water. The samples were then transferred to new plates and labeled according to their original concentration. Following enumeration, surviving organisms were subjected to length measurements.

Length Measurement

Measuring the length of live organisms was challenging due to their high mobility. To ease the process, two drops of diluted Lugol's solution (1:3 in distilled water) were added to immobilize the ostracods.

After 15 minutes under a fume hood, 0.1 ml of potassium thiosulfate was added to neutralize the excess iodine.

The organisms were then transferred onto a micrometer slide and visualized live under a microscope with a connected camera.

Photos were taken and lengths were measured using the ImageJ™ software.

The results were recorded in the laboratory logbook.

3.2.5.7 Final Chronic Toxicity Test of TBT, TMT, TPT

Dilutions for the Final Chronic Toxicity Test

Based on results of the preliminary test, four full chronic toxicity tests were conducted. Each

test included three concentrations of the tested substance in duplicate, along with a triplicate control.
All tested concentrations were prepared from [RR] using appropriate dilution ratios with EPA medium as the solvent.
Special care was taken to ensure that the lowest tested concentration produced no more than 20% mortality, while the highest concentration induced 80–100% mortality.
Filling of test wells, incubation, and result readout were performed identically to the preliminary test.

3.3 Acute Toxicity Test

The same crustaceans were used as in previous tests: Heterocypris incongruens.
The test aimed to assess 24-hour mortality using freshly hatched ostracods.
Unlike the chronic test, the organisms were not fed during the test and test vessels did not contain sediment. This ensured the elimination of factors affecting the bioavailability of lipophilic substances.
The toxicity endpoint was the LC50 parameter (Lethal Concentration for 50% mortality). Control trials without the test substance served as a reference for interpreting the results.
The acute toxicity test used the same equipment, materials, biological material, and media as the chronic Ostracodtoxkit F™ test.
The only modification was the use of 24-well test plates instead of 6-well plates.

3.3.1 Preliminary Acute Toxicity Test of TBT, TMT, TPT

Filling of Test Vessels, Incubation
A preliminary acute toxicity test was conducted for the compounds to determine an appropriate concentration range for the final test.
Each test included two replicates for six concentrations and three replicates for a negative control, resulting in 39 test wells in total. Each well contained 5 ml of the test solution (with the final well reduced by half).
Desired concentrations were obtained by serial two-fold dilution of [RR] using EPA medium as the solvent, following the dilution scheme outlined previously.
Each well was then inoculated with a specific, recorded number of organisms and incubated for 24 hours in a thermostatic incubator at 25°C.
Mortality
After 24 hours of incubation, live and dead organisms were counted under a microscope. Living organisms were those showing movement, while immobile ones were classified as dead. Results were entered into the lab logbook.

3.3.2 Final Acute Toxicity Test of TBT, TMT, TPT

Dilutions for the Final Acute Toxicity Test
Based on preliminary results, four full acute toxicity tests were performed. Each test consisted of six concentrations of the tested substance, prepared by two-fold serial dilution, with two replicates and a triplicate control.
All concentrations were prepared from [RR] in appropriate dilution ratios using EPA as the solvent.
When choosing the dilution ranges, the aim was to obtain the broadest possible spectrum of effects to ensure precise determination of the LC50 value.
Filling of test wells, incubation, and result reading were performed in the same way as in the preliminary test.
Mortality
The measured parameters in the Ostracodtoxkit F™ chronic toxicity test included mortality and organism length, whereas the acute toxicity test focused exclusively on mortality.
Mortality was expressed using the LC50 parameter (Lethal Concentration for 50% of the

population), which reflects the concentration at which 50% of the test organisms are expected to die.
Each test well yields a binary result—organisms either survive (negative outcome) or perish (positive outcome).
The ratio of dead individuals to the total number of organisms in each well was used to calculate the percent mortality.
With a set of mortality percentages from a given test, and through regression analysis or curve fitting, the LC50 value was calculated using Microsoft Excel™.
Organism Length
This parameter was assessed only for the chronic toxicity test and included: individual organism length, mean length, standard deviation, percent growth inhibition relative to the control.
Organism length was measured using the same method as described in section – Preliminary Chronic Toxicity Test – Result Readout.
Based on the lengths of test and control organisms, mean values and standard deviations were calculated.
From the average lengths, the percent growth inhibition relative to the control was also computed.

4. Results

4.1.Evaluation of the effect of oxygen conditions on ostracod length

An experiment was conducted to determine whether the absence of oxygen in incubated wells could affect the survival and body length of test organisms. Two test plates were prepared in which ostracods were placed in non-toxic sediment. One plate was covered only with a lid (allowing gas exchange), while the second plate was sealed with protective foil, preventing any gas exchange between the well content and the external environment.
Throughout the experiment, no mortality of ostracods was observed. The obtained body length results clearly indicate that there is no correlation between the presence or absence of oxygen and the final length of the organisms. Thus, it is acceptable to conduct tests in which each microplate well is tightly sealed, which protects the experimental setup from cross-contamination by volatile toxic substances migrating between wells.

4.2. OSTRACODTOXKIT F™ Test

Toxicity was assessed using the Ostracodtoxkit F™. Each test plate included 6 wells for three concentrations in duplicate. In each well, the following components were combined: 1 ml EPA solution, 10 freshly hatched ostracods, two scoops of sediment (1 ml), 1 ml algae (A = 3.0–3.6; λ = 678 nm), and 2 ml of an appropriately diluted working solution [WS] of the tested compound to achieve the target final concentration. In the control group, 2 ml of EPA solution was added instead of the test compound. Each plate was sealed with foil and incubated for 6 days. Mortality and body length were then recorded. The results were logged in the laboratory journal.
The following test effects were calculated: mortality and changes in ostracod length. At least three independent replicates were performed using different organism batches for each test..
After exposure to TBT, concentrations ≥4.0 ppm consistently caused 100% mortality. At 2.0 ppm, mortality varied between 30–100%; three replicates showed 100% mortality, two showed 30%, and one showed 60%. At concentrations ≤1.0 ppm, results were similar to control mortality. Therefore, the toxicity threshold for TBT is estimated at 1.0 ppm.
All tested concentrations of TMT ≥0.8 ppm caused 100% mortality. At 0.4 ppm, average mortality was 55% (excluding one outlier result of 100%). At concentrations ≤0.2 ppm, average mortality was 15%, comparable to the control. The toxicity threshold for TMT is estimated at 0.2 ppm.

For TPT, concentrations ≥2.0 ppm resulted in 100% mortality in every trial. Concentrations between 0.8–1.0 ppm produced variable mortality (5–100%): three results showed 100%, one 55%, and one 5%. Similarly inconsistent results were obtained for concentrations 0.4–0.5 ppm (mortality ranged from 10–100%). Results from April 17, 2018, are considered unreliable due to observable signs of decay (e.g., hydrogen sulphide odor upon removing the sealing foil). The cause of this reaction was not determined. Therefore, the toxicity threshold for TPT is estimated at 0.1 ppm.

Due to the high variability of the results, LC50 values for the compounds tested in the chronic Ostracodtoxkit F™ assay were not calculated; instead, toxicity was presented as percentage mortality.

The cause of the variability remains undetermined. Hypothesized explanations include: qualitative differences in the added sediment scoops, adsorption of the test compound on the pipette tip during transfer, variability in the amount or condition of algae used as food, potential damage to algae from ultrasonic homogenization prior to testing, which may have led to cellular decay, oxygen depletion, and increased organism stress, variability between cyst batches.

Further analysis is needed to identify the underlying cause.

Assessment of organism length changes

The average length of test organisms exposed to the compound was compared to that of the control group. The percentage growth inhibition was calculated.

Analysis revealed that TBT, at concentrations close to lethal (i.e., 2 ppm), significantly inhibited organism growth. Of five tests at 2 ppm TBT, two resulted in 100% mortality, while three showed 60% (one trial) and 30% (two trials) mortality. The average growth inhibition for the surviving organisms was 40.3%.

All TBT concentrations >2 ppm caused complete mortality, preventing length measurement. Concentrations ≤1 ppm showed inconsistent effects: one trial showed growth inhibition (11.5%), while another showed slight growth stimulation (-0.2%).

For TMT at 0.4 ppm, average growth inhibition was 39.7% (based on two out of three results, excluding one with 100% mortality). Results for concentrations ≤0.2 ppm were inconsistent: two showed strong inhibition (23.8% and 33.0%), while the other two showed minimal effects (5.4% and 12.3%), comparable to the control. All concentrations ≥0.8 ppm caused total mortality, preventing length assessment.

TPT at ≥2.0 ppm caused complete mortality, so length measurement was not possible. Data for 0.4–1.0 ppm were too variable to draw conclusions on growth inhibition.

Growth inhibition after six days of exposure in the Ostracodtoxkit F™ was observed only for TBT and TMT at concentrations roughly half of their lethal levels. For TPT, growth inhibition was mostly below 20% compared to controls. More data should be collected in future studies.

Control group assessment

No average mortality exceeding the threshold of 20% was observed in the control group throughout the experiments.

It is worth noting significant variation in average ostracod lengths between hatching batches, ranging from 585 to 814 μm. There were also high standard deviations within the same batch, e.g. the relative standard deviation was 18% (752±135).

The reason for this phenomenon remains unknown. It is hypothesized that it may be related to the quality of algae, sediment, or intraspecific variability in the cysts themselves.

4.3. Acute toxicity test

Acute toxicity was assessed using the procedures described previous. Each test plate included 12 wells. Each well contained a specific concentration of the test compound, obtained by diluting the working solution [WS], and a precisely transferred number of test organisms. After tightly sealing the plates, a 24-hour incubation followed. Mortality was recorded, and

LC50 values were calculated. At least three independent replicates were performed, each using a different organism batch..
Data analysis indicated that tributyltin chloride (TBTCl) was the most toxic compound, with an LC50 of 0.113 ppm, while trimethyltin chloride (TMTCl) was the least toxic, being 15 times less potent, with an LC50 of 1.76 ppm. Triphenyltin chloride (TPTCl) showed similar toxicity to TBTCl, with an LC50 of 0.167 ppm.
The lower toxicity of TMTCl may be due to its higher volatility, which is likely a result of the lower molecular weight of methyl substituents.
Control group mortality
No mortality was observed in the control group during the entire experiment.

4.4. Comparison of acute and chronic toxicity results

Among the tested organotin compounds, TPT exhibited the strongest chronic toxic effect (mortality) at concentrations above 0.1 ppm. TMT showed moderate chronic toxicity, while TBT was the least toxic in chronic tests (threshold >1 ppm).
In acute toxicity tests, TBT and TPT were both significantly more toxic than TMT, with TMT's LC50 being over 15 times higher.
However, direct comparison of chronic and acute toxicity is challenging due to major differences in test protocols, including exposure time, presence of sediment, and availability of food. Notably, TPT toxicity remained similar in both test types. In contrast, TMT showed increased toxicity with prolonged exposure, while TBT was more toxic in the acute test—possibly due to reduced bioavailability in the chronic setup, as TBT may bind strongly to sediment.
Unfortunately, a full bioavailability analysis was beyond the scope of this project.

5. Conclusions

1. The highest acute toxicity for ostracods was exhibited by tributyl tin chloride, with an LC50 of 0.113 ppm. A slightly less toxic substance was triphenyl tin chloride (LC50 = 0.167 ppm). In contrast, trimethyl tin chloride proved to be several times less toxic than TBT and TPT, with an LC50 value of 1.76 ppm.
2. In the chronic toxicity test using the Ostracodtoxkit F™ method, the lethal effect (organism death) was caused by the same concentration of triphenyl tin chloride (above 0.1 ppm). However, different results were obtained for tributyl tin chloride – mortality was observed at concentrations above 1 ppm.
3. A decrease in the length of ostracods after 6 days of the Ostracodtoxkit F™ test was observed only for TBT and TMT at concentrations half of the lethal concentrations.
4. In the case of TPT, the obtained results of growth inhibition were, in most cases, below 20% compared to the control.
5. No correlation was found between the presence or absence of oxygen and the length of incubated ostracods. Therefore, it is possible to test volatile substances by completely sealing the test vessels.
6. The test effect for ostracods (Heterocypris incongruens) occurs at much higher concentrations than the environmental water concentration of TBT.

Disclosure

Author's contribution:
Conceptualization: TS
Methodology: TS
Software: TS, MK, HB, MM, IK
Check: TS, PK, PM, HB, IK
Formal analysis: TS, HB, MM

Investigation: TS
Resources: TS, MK, HB
Data curation: TS, PK, MM, JN
Writing -rough preparation: TS, PM, MB
Writing -review and editing: TS, HB, MM, JN
Visualization: TS, PM, MB
Supervision: TS, PM, MB, JN, IK
Project administration: TS, HB
All authors have read and agreed with the published version of the manuscript.

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