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Bioaccumulation of Triorganotin Compounds by a Sediment-Dwelling Organism (*Chironomus riparius*): Assessment of Bioavailability, Uptake and Elimination Processes

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Abstract

Triorganotin compounds (TOTs), in particular tributyltin (TBT) and triphenyltin (TPT), are recognized as compounds with high toxicity towards aquatic organisms and populations. Due to their widespread use as antifouling agents in boat paints, and as fungicides for wood and crop protection, they are considered as contaminants of global concern. Despite worldwide restrictions in the use of TOTs as antifouling agents in boat paints, high concentrations may persist in sediments which act as ultimate sinks for TOTs in aquatic environments. Reports on total TOT concentrations in sediments are abundant, but little is known about the concentrations actually available for the organisms. The impacts of TOTs on sediment-dwellers are therefore difficult to predict. This work focuses on the fate of TOTs in a representative freshwater sediment organism and on the bioavailability of TOTs.

Midge larvae *Chironomus riparius* were exposed to TBT and TPT solutions to gain insight into the mechanisms of bioconcentration and into important factors that determine the bioavailability. In order to obtain quantitative toxicokinetic data for TBT and TPT, the laboratory culture of *Chironomus riparius* larvae was optimized for maximum densities. A fast analytical technique with high accuracy and precision was developed for the determination of organotin compounds in biota samples of small size, using gas chromatography/mass spectrometry (GC/MS).

The bioconcentration experiments were conducted at pH 5 and pH 8. A pH of 5 was chosen, because the positively charged TBT^+ and TPT^+ species predominate, and the conditions are still reasonable for *Chironomus* larvae. At pH 8, more than 98 % of the TOTs are present in the neutral TBTOH and TPTOH form, respectively. A two-pool model could successfully describe both uptake and elimination data in simultaneous fits, and uptake, excretion, and metabolism rate constants could be derived with high reproducibility for different batches of larvae. Bioconcentration was found to be significantly lower for TBT than for TPT, although TBT is more hydrophobic. The major reason was the rapid metabolism of TBT to DBT (dibutyltin) and further to MBT (monobutyltin) with similar rates at pH 5 and pH 8. If the metabolism rate constants were set equal to zero, the model

calculations yielded steady state bioconcentration factors that reflected the relative hydrophobicities of TBT and TPT. No significant metabolism was found for TPT, suggesting a higher biomagnification potential as compared to TBT.

The bioconcentration of both TBT and TPT was higher at pH 8 than at pH 5 but for TPT, the difference between both pH's was much less pronounced than for TBT. It was concluded that the charged TPT^+ species was taken up to almost the same extent as the neutral TPTOH species, whereas the uptake of TBT^+ was almost one order of magnitude smaller as compared to TBTOH, although not negligible. These observations were explained with the stronger affinity of TPT^+ to oxygen ligands.

The association of TBT and TPT with dissolved organic matter such as Aldrich humic acid (AHA) is known to be pH-dependent and the AHA-water distribution ratios (D_{AHA}) exhibit a maximum at the pH corresponding to the pK_a of the specific compound. Apparent D_{AHA} values were derived from bioconcentration experiments in the presence of AHA and were compared with D_{AHA} values determined in dialysis experiments. Except for some explainable discrepancies, a good agreement between both sets of D_{AHA} values was found, confirming the hypothesis that TOTs associated with AHA are not bioavailable.

Chironomus larvae exposed to a reference sediment showed dark coloured guts after only few hours, indicating significant uptake of sediment particles. The comparison of the TBT bioaccumulation in sediment- and water-exposed larvae showed that uptake from the pore water and uptake via sediment ingestion were of similar magnitude. It was concluded that the bioavailable TBT fraction in sediments is not limited to the freely dissolved TBT in the pore water but comprises also particle-bound TBT.

Chironomus riparius was found to be very well suited to study the speciation and the bioconcentration of TOTs. Using a two-pool model, the fate of butyl- and phenyltin compounds in the larvae could successfully be described. This model offers a promising tool for assessing the uptake, elimination, and the bioavailability of other contaminants than organotin compounds to *Chironomus* species or other benthic organisms.

Zusammenfassung

Triorganozinnverbindungen (TOTs), insbesondere Tributylzinn (TBT) und Triphenylzinn (TPT) gelten als hochgiftige Substanzen für Waserorganismen. Sie werden weltweit angewendet in Antifouling-Farben für Bootsanstriche sowie als Fungizide für Holzschutz und in der Landwirtschaft. In vielen Ländern wurde der Einsatz von organozinnhaltigen Antifouling-Farben eingeschränkt oder verboten. Sedimente wirken jedoch als Senken für TOTs, so dass auch nach der Inkraftsetzung von Verboten teilweise beträchtliche Konzentrationen anzutreffen sind, wie in vielen Publikationen nachzulesen ist. Welcher Anteil der insgesamt vorhandenen TOTs aber tatsächlich von den Organismen aufgenommen werden kann, ist weitgehend unbekannt. Demzufolge ist es sehr schwierig abzuschätzen, welche Auswirkungen auf Sedimentlebewesen effektiv zu erwarten sind. Im Zentrum dieser Arbeit stehen daher die Fragen nach der Bioverfügbarkeit und nach dem Verbleib von TOTs in typischen Sedimentorganismen.

Larven der Zuckmücke *Chironomus riparius* wurden in Lösungen mit TBT und TPT exponiert, um die Biokonzentration und die Bioverfügbarkeit zu untersuchen. Um eine möglichst hohe Zahl an Testorganismen zu erhalten, wurde eine Laborzucht mit *Chironomus riparius* Larven etabliert und optimiert. Ferner wurde eine sehr rasche und präzise Analysenmethode entwickelt für die Bestimmung von Organozinnverbindungen in kleinen Organismen, unter Verwendung von Gaschromatographie gekoppelt mit Massenspektrometrie (GC/MS).

Die Biokonzentrationsexperimente wurden bei pH 5 und bei pH 8 durchgeführt. Die Motivation für pH 5 lag darin, dass hier die positiv geladenen TBT^+ und TPT^+ Spezies vorherrschen, aber die Bedingungen für die *Chironomus* Larven noch zumutbar sind. Bei pH 8 liegen mehr als 98 % der TOTs in der neutralen TBTOH beziehungsweise TPTOH Form vor. Mit einem Zwei-Pool Modell wurden sowohl die Aufnahme- als auch die Eliminationsdaten mit simultanen Kurvenfittings beschrieben. Daraus konnten die Geschwindigkeitskonstanten für Aufnahme, Exkretion und Metabolismus reproduzierbar abgeleitet werden. Obwohl TBT hydrophober als TPT ist, wurde eine signifikant geringere Biokonzentration festgestellt.

Der Hauptgrund liegt in einem sehr raschen Abbau zu DBT (Dibutylzinn), und weiter zu MBT (Monobutylzinn). Bei TPT wurde kein nennenswerter Metabolismus festgestellt, so dass im Vergleich zu TBT ein höheres Biomagnifikationspotential zu erwarten ist.

Das Ausmass der Biokonzentration war sowohl für TBT als auch für TPT bei pH 8 höher als bei pH 5, jedoch war bei TPT der Unterschied zwischen den beiden pH-Werten weniger ausgeprägt: Die geladenen TPT^+ Spezies wurden fast in gleichem Ausmass wie die neutralen TPTOH Spezies aufgenommen. Im Gegensatz dazu war die Aufnahme von TBT^+ beinahe um eine Zehnerpotenz kleiner als die Aufnahme von TBTOH, jedoch nicht vernachlässigbar. Dieser Befund war durch die höhere Affinität von TPT^+ zu Sauerstoffliganden zu erklären.

Die Wechselwirkungen von TBT und TPT mit gelöstem organischem Material, zum Beispiel mit Aldrich Huminsäure (AHA), ist stark pH-abhängig und die AHA-Wasser Verteilungskonstanten (D_{AHA}) sind maximal bei einem pH, der dem pK_a der Verbindung entspricht. Aus den Biokonzentrationsexperimenten mit AHA wurden rechnerisch D_{AHA}–Werte abgeleitet, die mit experimentell bestimmten D_{AHA}–Werten aus Dialyse-Experimenten verglichen wurden. Die Übereinstimmung zwischen den beiden Datensets war mehrheitlich gut. Dies bestätigt die Hypothese, dass an AHA gebunden TOTs nicht bioverfügbar sind.

In einem Referenz-Sediment exponierte *Chironomus* Larven hatten nach wenigen Stunden die Därme gefüllt mit Sedimentpartikeln. Die Auswertung der TBT Konzentrationen ergab, dass etwa in gleichem Ausmass TBT via Sediment-Partikel und aus dem Porenwasser aufgenommen worden war. Daraus folgt, dass die Bioverfügbarkeit von TBT in Sedimenten nicht ausschliesslich eine Frage der Porenwasserkonzentration, sondern auch des partikulär gebundenen TBT ist.

Die Chironomus riparius Larven erwiesen sich als sehr geeignete Sensoren für die Speziierung der TOTs. Unter Anwendung eines Zwei-Pool Modells konnte der Verbleib der Butyl- und Phenylzinnverbindungen in den Larven adäquat beschrieben werden. Dieses Modell ist ein vielversprechendes Werkzeug zum Studium von Aufnahme, Elimination und Bioverfügbarkeit verschiedener Schadstoffe mit *Chironomus* Arten oder anderen benthischen Organismen.

1

General Introduction

Over the past 40 years, organotin compounds have become important as PVC stabilizers and biocides in various applications [1]. Based on their widespread use and their high toxicity, they are considered as contaminants of global concern. In aquatic ecosystems, the triorganotin compounds tributyltin (TBT) and triphenyltin (TPT) are of particular relevance. TBT is released into aquatic environments mainly by its use as antifouling agent, where the globally released amounts are actually estimated at 3000 tons per year [2]. Although TPT has been used as cotoxicant in some antifouling paints up to 1985 [1], the major application is crop protection [1, 3], where globally 3000 tons per year are used [2].

The extremely high toxicity of TBT to aquatic organisms has been discovered already in the early eighties [4, 5]. The shell deformations of oysters *Crassostrea gigas* in the Bay of Arcachon (France) [5] and their impact on economy led to a worldwide concern and induced intensive research on the TBT problem [6-10]. To date, numerous mechanisms of toxic action have been reported for TBT, such as disturbance of cell membrane structures [11], inhibition of the energy production in cells [12, 13], disturbance of the cells calcium concentration control [14, 15], and inhibition of P450–dependent enzymes [16] with the possible result of endocrine disruption [4]. A comprehensive review is given in ref. [17].

Several of the TBT-related effects have also been demonstrated for TPT [12, 18, 19].

Based on the well known hazard potential, many countries have regulated the use of TBT as antifouling agent and banned TBT for boats shorter than 25 m in length [20, 21]. As a result of these regulations, lower TBT concentrations in freshwater marinas and coastal marine ecosystems were recently reported [22-24]. In a most recent study [25], moderate risks for aquatic ecosystems were attributed to the actually present organotin concentrations, and a controversial discussion about the usefulness of a total TBT ban is currently taking place [26, 27]. However, sediments act as the ultimate sinks for triorganotin compounds in aquatic ecosystems, and since the estimated half-lifes of TBT in sediments range from months [28] to several years [29-31], high concentrations may persist in heavily contaminated sediments for a long time, even after a total TBT ban for boat paints. Furthermore, TPT will still be used in the agricultural field, where no regulations have been established to date.

Despite the sound knowledge on toxic effects to aquatic organisms and populations, and the numerous recent reports on triorganotin concentrations in sediments [30-37], the impacts on sediment-dwelling organisms are very difficult to predict. Little is known about the actually bioavailable organotin concentrations in the sediments. For example, organisms which burrow in the sediment will take up TBT or TPT from the sediment pore water, but TBT and TPT associated with the dissolved organic matter (DOM) of the pore water may not be bioavailable. Furthermore, for some sediment-dwellers different uptake routes may simultaneously be important, such as uptake from water and uptake via ingestion of particles. In view of these facts, it is somewhat surprising that only very few studies have adressed specifically the bioavailability of the organotin compounds under different environmental conditions [38, 39].

This thesis wants to contribute to fill the gap between the known total triorganotin concentrations in environmental compartments and the actually bioavailable concentrations. The general goal was to investigate important factors that determine the bioavailability of TBT and TPT in aquatic ecosystems, particularly in sediments.

The specific objectives of this work were (1) to compare the bioconcentration of triorganotin compounds in freshwater organisms of different ecological niches and to check the suitability of a representative sediment organism for bioavailability studies, (2) to study the influence of pH on TBT and TPT bioavailability, (3) to quantify the bioavailable TBT and TPT fractions in the presence of dissolved organic matter (DOM), using Aldrich humic acid as model DOM, (4) to establish a model for triorganotin uptake and elimination in a representative freshwater sediment organism (midge larvae *Chironomus riparius*), and (5) to study the importance of different uptake routes for the triorganotin uptake by *Chironomus riparius* in a reference sediment.

The results of this work are presented in the following way:

Chapter 2 shows a comparison of TBT and TPT bioconcentration in three different freshwater organisms, i.e., a zooplankton species (*Daphnia magna*), sediment-dwelling insect larvae (*Chironomus riparius*) and fish yolk sac larvae (*Thymallus thymallus*). This preliminary study demonstrated the suitability of *Chironomus riparius* as sediment organism for bioavailability studies, and formed the base for optimizing the follow-up experiments.

In Chapter 3, a new analytical technique for the determination of organotin compounds in biota samples of small size, such as *Chironomus* larvae, is presented. The high accuracy and precision of this fast and sensitive method provided the base for the quantitative description of the processes that determine triorganotin uptake and elimination in *Chironomus riparius*.

Chapter 4 describes the uptake and elimination of TBT and TPT in *Chironomus riparius* larvae in the absence and presence of Aldrich humic acid (AHA). The fate of the triorganotin compounds in *Chironomus* larvae is qualified by a model that covers simultaneously all relevant uptake and elimination processes.

Chapter 5 presents the results of experiments, where *Chironomus* larvae were exposed to a well characterized reference sediment (PACS-2) with certified butyltin concentrations. The relative importance of TBT uptake from water and via sediment particles is addressed.

In Chapter 6, general conclusions are drawn and the environmental significance of the results is discussed.

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Bioconcentration and Bioavailability of Organotin Compounds: Influence of pH and Humic Substances

Bioconcentration of triphenyltin (TPT) and tributyltin (TBT) was studied in the freshwater organisms *Daphnia magna* (zooplankton), *Chironomus riparius* (sediment organism), and *Thymallus thymallus* (fish yolk sac larvae). TPT bioconcentration factors (BCF) at pH 8 were highest for *Thymallus* (2200), followed by *Chironomus* (680) and *Daphnia* (190). The differences could not be fully explained by different total lipid contents. Metabolism and lower bioconcentration were observed for tributyltin TBT in *Chironomus*. The BCF's of both TBT and TPT were higher at pH 8 than at pH 5, but the difference was much less pronounced than predicted by the octanol-water partition model. This suggests that besides the hydroxide species (TBTOH and TPTOH), the cations (TBT^+ and TPT^+) are also taken up by the organisms and that the octanol-water partition model underestimates the uptake of the charged species. Low concentrations of humic substances (HS) did not reduce the bioconcentration of TPT in *Daphnia* and *Thymallus*. A significant reduction of TPT bioavailability

occurred only at relatively high concentrations of HS (> 10 mg C L^{-1}). The results of this study provide an important base for future investigations aiming at a better understanding of the fate of TBT and TPT in freshwater foodwebs.

2.1 Introduction

Organotin compounds are among the most hazardous pollutants in aquatic ecosystems [1]. Tributyltin (TBT) and triphenyltin (TPT) are of particular interest because of their widespread use as biocides. TBT is an important biocide in antifouling paints. The ecotoxicological hazards associated with TBT [2-7] have resulted in restrictions on its use in many countries. In spite of these regulations, the release of TBT into aquatic ecosystems persists due to its use in antifouling paints on large vessels, paint removal from pleasure boats, application of TBT in wood preservation and resuspension from contaminated sediments [8-11]. Although TPT has been used as cotoxicant with TBT in some antifouling paints, the major application of TPT is in agriculture [12]. TPT is used as a fungicide for various crops and enters aquatic ecosystems mainly via leaching and runoff from agricultural fields. Tetrabutyltin (TeBT) occurs as a byproduct of the production of other organotin compounds. Although the amounts of TeBT released into aquatic environments are globally of minor importance, some TeBT contaminated areas have been described [13]. The ecotoxicological consequences of this contamination, however, are not known.

With respect to the ecotoxicology of organotin compounds, many studies on the contamination of aquatic systems and the toxicity have been performed [1-7, 14, 15]. Knowledge has also been gained on the distribution pattern of organotins in environmental compartments [12, 16-19]. However, the long-term ecotoxicological effects of organotin contaminants on the structure and function of aquatic ecosystems are still not well understood, particularly with respect to biomagnification in food webs [12, 20-22] and toxicity of contaminated sediments. These hazards are dependent on the bioavailability of the contaminants.

The bioavailability of environmental chemicals can be studied by assessing the bioconcentration with bioaccumulation experiments. Several general factors such as hydrophobicity, speciation and organism-specific properties determine the degree of bioconcentration. For neutral organic compounds of medium hydrophobicity and low biotransformation potential, octanol-water partition coefficients (K_{ow}) can serve as an estimate of the maximum possible bioconcentration in organisms [23-25]. For chemicals that can form charged species in water, different bioconcentration of the charged and uncharged species should be expected. In many cases, however, simple partitioning of the compound between the aqueous phase and the organisms can not be assumed. Species-specific factors such as uptake efficiency, growth dilution, metabolism and excretion can modify the degree of bioconcentration.

Organotin compounds, in particular TBT and TPT, differ in their properties from neutral organic compounds. The speciation of TBT and TPT shows a strong pH-dependence [26]. Both compounds are present as cations at low pH and as hydroxides at higher pH. While they can also form complexes with other anions, these species are only of minor importance in typical freshwater systems. Therefore, only the hydroxide species (TBTOH / TPTOH) and the cations (TBT^+ / TPT^+) have to be considered. The two species, however, exhibit very different partitioning and sorption behavior. The octanol-water distribution ratios (D_{ow}) of TBT and TPT are more than an order of magnitude higher at pH 8 than at pH 3 [26]. This is related to the fact that TBTOH and TPTOH, but not TBT^+ and TPT^+ readily partition into the octanol phase [26]. For partitioning and sorption into humic substances (Aldrich HS), both TBT and TPT have a maximum distribution ratio D_{OM} of approximately 140,000 L kg_{OM}^{-1} between pH 5 and 6 [27].At typical ambient conditions (pH 8), the D_{OM}-values are considerably lower for both TBT and TPT (10,000 - 15,000 L kg_{OM}^{-1}) [27].

In a previous study, TBT bioconcentration in *Daphnia magna* was found to be higher at pH 8 than at pH 6 [28]. In the same study, Aldrich humic substance (HS) reduced the bioconcentration of TBT in *Daphnia magna.* In the present work, we have extended our investigations on the bioavailability of organotins to different freshwater organisms and different compounds. The major goals were

(1) to compare the bioconcentration of TPT by three different organisms representing different ecological niches,

(2) to verify whether the pH-dependent speciation of TBT and TPT results in different bioconcentration factors at higher and lower pH, and

(3) to study the effect of relatively low concentrations (<10 mg L^{-1}) of Aldrich humic substance (HS) on the bioconcentration of TPT.

We report experimentally determined bioconcentration factors for TPT in *Daphnia magna* (zooplankton), *Chironomus riparius* (sediment organism) and *Thymallus thymallus* (fish yolk sac larvae). Bioconcentration of TBT, TPT and TeBT in *Chironomus riparius* at pH 8 and pH 5 is compared, using TeBT as reference compound that may only undergo hydrophobic partitioning. Finally, results on the bioconcentration of TPT in *Daphnia* and *Thymallus* in presence of Aldrich humic substance (HS) are presented.

2.2 Materials and Methods

2.2.1 Chemicals

Tributyltin chloride TBTCl (> 97 %), dibutyltin dichloride $DBTCl_2$ (~97 %), triphenyltin chloride TPTCl (> 97 %) and tetrabutyltin TeBT (~98 %) were obtained from Fluka Chemie AG (Buchs, Switzerland). Diphenyltin dichloride $DPTCl_2$ (> 98 %) and tripropyltin chloride (~98 %) were purchased from ABS (Basel, Switzerland). Butyltin trichloride $MBTCl_3$ (~95 %), phenyltin trichloride $MPTCl_3$ (~98 %) and tripentyltin chloride (~96 %) were supplied by Aldrich, Steinheim (Germany). Solutions of ethylated butyl- and phenyltin compounds in methanol (~100 mg Sn L^{-1}) were stored in the dark at +4 °C as primary standards. For external calibration, the primary standards were diluted with hexane. Internal standards were prepared by diluting tripropyltin chloride and tripentyltin chloride with acetone. Stock solutions of tributyltin chloride, triphenyltin chloride and tetrabutyltin in acetone were used to spike experimental media.

Aldrich humic substance (HS) as Na-humate was used without further purification. HS is similar to humic substances in many wetlands [29]. Although HS may not exactly represent dissolved humic and fulvic acids of many freshwater ecosystems, it is appropriate for investigations of the general behavior of dissolved humic substances and the results can be compared to results of other studies [28, 30-33]. A HS stock solution for the experiments was obtained by dissolving 2.5 g HS in 5 L of nanopure water, centrifuging at 8,000 g for 30 min and filtering through a 0.45 µm filter.

2.2.2 *Daphnia* Experiments

Daphnia magna were cultivated according to the OECD Guideline 202 [34] in synthetic medium M7 [35] and fed with the green algae *Scenedesmus subspicatus*. The major cations in this medium are Ca^{2+} (2 mM), Mg^{2+} (0.5 mM) and Na^{+} (0.8 mM), while the most important anions are HCO_3^- (0.8 mM), SO_4^{2-} (0.5 mM) and Cl^- (4 mM). The ionic strength of the medium is 13.5 mM. The *Daphnia* were cultured in a 20 ± 1 °C climate chamber with a 16:8 hours light:dark cycle. The experiments were conducted under the same conditions using 21±2 d old *Daphnia* that were not fed during the experiment. Table 2-1 gives an overview of the experimental conditions. In each test, 80 *Daphnia* were exposed in 1 L glass beakers, containing 800 mL of test solution with TPT. The pH was adjusted to 8.0 at the beginning of the experiment with 0.5 M NaOH, monitored twice a day, and readjusted with 0.5 M NaOH if necessary. Beakers were not aerated. Oxygen saturation was measured at the beginning and end of each experiment and ranged from 78.4 to 86.6 %. After 0, 8, 24, 48 and 72 h, 10 individuals were removed. *Daphnia* were rinsed with nanopure water, dried on cellulose filters, weighed and transferred to glass vials for storage at -20°C until analysis. At 0 h and 72 h, aliquots of experimental medium were taken from each glass beaker, acidified to pH 2 and stored at 4 °C for organotin and DOC analyses. In the experiments with humic substance (HS), aliquots of the HS stock solution were added to the experimental medium, resulting in DOC concentrations of 1.1 - 14.2 mg C L^{-1}. Three replicates were made for every DOC concentration.

2.2.3 *Chironomus* Experiments

Egg masses of non-biting midge *Chironomus riparius* were received from Springborne Laboratories AG (Horn, Switzerland). The larvae were reared as described by Streloke and Köpp [36] in 10 L full glass aquaria, containing precleaned and shredded paper towels as substrate and synthetic medium M7 [35] as overlying water. The water column was aerated at a rate of 1 - 2 bubbles per second and the larvae were fed with fish food Tetramin®. The experiments were conducted in a climate chamber at 20 ± 1 °C with a 16:8 hours light:dark cycle. 80 two week old *Chironomus* larvae were exposed in 1 L glass beakers containing 800 ml of M7 medium with TBT, TPT or TeBT (Table 1). The larvae were not fed and the containers were not aerated during the experiments. Oxygen saturation was determined at the beginning and at the end of the experiments. The mean values were 67.1 ± 5.7 % and 64.6 ± 4.3 %, respectively. The pH was measured three times a day and adjusted to the desired values of 8.0 and 5.0 with 0.5 M NaOH and 1.0 M HCl, respectively. After 6, 24, 48 and 72 hours, samples of 10 *Chironomus* larvae were removed, rinsed, dried on filter paper, weighed into vials and stored at -20 °C for organotin analyses and lipid determination. The TBT and TPT experiments were replicated 5 times and the TeBT experiment had 6 replicates.

Table 2-1 Experimental conditions

Organism	Compound	Exposure concentration (c_w) [µg L^{-1}]	pH	Temperature [°C]	Wet weight [mg]	Dry weight [% of wet weight]	Lipid content [% of wet wgt.]	Control mortality [%]
Daphnia	TPT	8.0 ± 1.4 (15)	8.0	20 ± 1	2.1 ± 0.4 (60)	7.1 ± 0.5 (14)	0.3 ± 0.1 (14)	20 ± 11 (15)
Chironomus	TPT	4.3 ± 0.5 (5)	5.0	20 ± 1	5.5 ± 1.1 (40)	12.0 ± 1.0 (7)	0.6 ± 0.1 (7)	3.8 ± 3.5 (5)
Chironomus	TPT	4.8 ± 0.4 (5)	8.0	20 ± 1	(-)	(-)	(-)	(-)
Chironomus	TBT	7.1 ± 0.8 (5)	5.0	20 ± 1	4.4 ± 0.8 (40)	(-)	(-)	9.0 ± 5.0 (5)
Chironomus	TBT	6.5 ± 0.9 (5)	8.0	20 ± 1	(-)	(-)	(-)	(-)
Chironomus	TeBT	1.6 ± 0.3 (6)	5.0	20 ± 1	4.5 ± 0.5 (40)	(-)	(-)	4.9 ± 2.7 (6)
Chironomus	TeBT	1.7 ± 0.6 (6)	8.0	20 ± 1	(-)	(-)	(-)	(-)
Thymallus	TPT	3.2 ± 0.4 (10)	8.3	15 ± 1	18.9 ± 0.9 (40)	21.1 ± 4.0 (12)	3.1 ± 0.5 (12)	4 (1)

Data are given as mean ± SD. Numbers in parentheses indicate the number of independent measurements. The exposure concentration (c_w) refers to the geometric mean of the concentration at the beginning of the experiment and after 72 hours (*Daphnia*, *Chironomus*) or 48 hours (*Thymallus*), respectively. (-) not determined. With the exception of tetrabutyltin, all exposure concentrations refer to the respective butyltin and phenyltin chlorides. The factors for converting the reported concentrations into µg Sn L^{-1} are 0.42, 0.39, 0.36, 0.34, 0.39, 0.35, and 0.31 for MBT, DBT, TBT, TeBT, MPT, DPT, and TPT, respectively.

2.2.4 Fish Larvae Experiments

Fertilized eggs of graylings, *Thymallus thymallus,* from the Rhine river were transported to the laboratory and adapted to aerated groundwater in a climate chamber at 15 ± 1 °C in the dark for three days. The groundwater had a total hardness of 6.8 meq L^{-1} (Ca^{2+} + Mg^{2+}), an alkalinity of 5.8 meq L^{-1} and an ionic strength of 14.9 mM. DOC, organotins, nitrite and ammonia were not present at detectable levels. Experiments were performed with freshly hatched yolk sac larvae in the same climate chamber, in the dark. In 10 L full glass aquaria, 250 larvae were exposed for up to 168 h in 3 L groundwater with TPT (Table 2-1). The larval density was 1.3-2.1 $g \cdot L^{-1}$. Aquaria were aerated, and O_2 and pH determined daily. The average pH was 8.3 ± 0.1 and all measured oxygen saturation values were ≥ 97%. No food was provided. The experimental water was changed every 48 h, and 250 mL aliquots were removed at the beginning of the experiment and prior to water renewal. Corresponding water samples were pooled, acidified to pH 2 and stored at 4 °C for determination of DOC and organotins. The experimental set-up included one test with TPT and no humic substance (HS), and tests with TPT plus 1.0, 1.7, 4.3 and 8.8 mg C L^{-1} HS. After seven different time points 20-30 fish larvae were removed, rinsed, dried on aluminium foil, weighed and stored at -20 °C until analysis. The experimental conditions are summarized in Table 2-1.

2.2.5 Organotin Analysis

Butyltins (MBT, DBT, TBT, TeBT) and phenyltins (MPT, DPT, TPT) in water and biological samples were determined according to methods described elsewhere [18, 37] with slight modifications. The acidified water samples (pH 2) were transferred into 50 mL volumetric flasks, spiked with the internal standards tripropyltin and tripentyltin chloride, and extracted with 2 portions of 0.25 % tropolone solution in pentane/diethylether (2 : 3) and 1 portion of pentane. The combined organic phases were dried on anhydrous $CaCl_2$, concentrated to 1 ml under a gentle nitrogen stream and ethylated with 2M ethylmagnesium bromide in tetrahydrofurane. After 10 min, the excess of Grignard reagent was hydrolyzed by dropwise addition of 1 M HCl. The top layer was removed and concentrated under nitrogen to 2 ml for GC analysis. Biological samples were acidified to pH 2 with HCl,

homogenized and spiked with tripropyltin and tripentyltin chloride. The extraction and derivatization procedure was the same as for water samples, but the extracts were purified by adsorption chromatography on silicagel (0.5 g of silicagel in a pasteur pipet) and subsequent elution with hexane. The hexane extracts were analyzed using a Carlo Erba HRGC 5160 gas chromatograph with split/splitless injector, a flame photometric detector (SSD 250) and a DB-5 column (J&W) with i.d. 0.32 mm and a 0.25 µm film. Recovery rates refering to tripropyltin and tripentyltin chloride were routinely assessed in every sample and ranged from 51 to 81 % and from 55 to 69 % in water and biological samples, respectively. The relatively low overall recovery rates are mainly due to the fact that the whole analytical procedure was covered by this internal standardization method. The reported recoveries include reduced extraction efficiency in water samples with humic substances as well as slight evaporation losses in the concentration steps and sorption to silicagel (phenyltin compounds in biological samples). The detection limits for butyltin and phenyltin compounds ranged from 0.09 to 0.35 µg L^{-1} in water samples and from 24 to 139 ng g^{-1} in biota. With the exception of tetrabutyltin, all results in this paper refer to the respective butyltin and phenyltin chlorides.

2.2.6 DOC Analyses

Water samples were membrane filtered (0.45 µm) and DOC was determined by high temperature combustion followed by infrared detection of CO_2 using a Shimadzu TOC-5000A total organic carbon analyzer. In the controls without HS, DOC concentrations of 1.5 mg C L^{-1} (medium M7) and 1.7 mg C L^{-1} (groundwater) were detected at the end of the experiments. These residues were subtracted from the measured DOC, as they originate partly from the acetone in the spiked stock solutions and partly from exudates of the test organisms.

2.2.7 Determination of Total Lipid Content

The total lipid content in *Daphnia*, *Chironomus* and *Thymallus* samples was determined following the sulphophosphovanillin method of Barnes and Blackstock [38] with specific adaptations for small organisms [39].The lipids were extracted from the lyophilized samples with hexane/isopropanol (3:2) and hydrolysed with sulfuric acid. After reaction with vanillin in

concentrated orthophosphoric acid for 90 min, the absorbance at 528 nm was measured with a Hitachi U-1000 UV/Vis spectrophotometer. The calibration standards were obtained by treating triolein standards in the same way as the samples.

2.2.8 Data Analysis

The organotin uptake curves were fitted through the experimental data points with the integrated equation of the 1-Box model [40-42] . The model yielded the uptake rate constant k_1 and the elimination rate constant k_2 as fitted parameters. Individual bioconcentration factors were calculated for each replicate and the differences between different pH values and different concentrations of humic substance were tested for statistical significance with a Student t-test (two-sided, significance level p = 0.05).

The following definitions and equations were used:

1. 1-Box model, integrated form: $\frac{c_b}{c_w} = \frac{k_1}{k_2} \cdot \left(1 - e^{-k_2 \cdot t}\right)$

   k_1 = uptake rate constant [mL g^{-1} h^{-1}]

   k_2 = elimination rate constant [mL g^{-1} h^{-1}]

   c_b = concentration in biota [ng g^{-1}]

   c_w = exposure concentration [ng g^{-1}] or [ng mL^{-1}]

   c_w was calculated as the geometric mean of the concentration at the beginning of the experiments and the concentration after 48 (*Thymallus*) or 72 hours (*Daphnia, Chironomus*).

2. BCF: bioconcentration factor at the end of the experiment.

$$BCF = \frac{c_b}{c_w}$$

All BCF's reported in this study are experimentally determined and refer to the end of the experiments, during which steady state was not necessarily achieved.

2.3 Results and Discussion

2.3.1 Bioconcentration at pH 8

The concentrations of organotins in the exposure waters decreased in all of the experiments. The TPT concentration decreased in the experiments with *Daphnia* from 8.6 ± 1.7 to 7.4 ± 1.6 µg L^{-1}, with *Chironomus* from 6.3 ± 0.4 to 3.1 ± 0.6 µg L^{-1} and with *Thymallus* from 4.2 ± 0.2 to 2.4 ± 0.5 µg L^{-1}. In the TBT- and TeBT-experiments with *Chironomus*, the concentrations declined from 10.0 ± 1.0 to 4.7 ± 0.8 µg L^{-1} and from 3.1 ± 0.5 to 0.9 ± 0.4 µg L^{-1}, respectively. Taking into account the organotin concentrations in water and biota, no quantitative mass balance was achieved. Losses may be caused mainly by absorption to the glass wall. The analyses of water samples from control experiments showed, however, that the decrease of the concentrations occurred in a time-dependent, non linear fashion (data not shown). Thus, the geometric mean of the measured initial and final concentrations gives realistic values for the average exposure concentrations (Table 2-1).

The uptake of TPT was species dependent. Considerable differences occurred among *Daphnia* (pH 8.0), *Chironomus* (pH 8.0) and *Thymallus* (pH 8.3) as shown in Figure 2-1. A steady-state was reached in the experiment with *Daphnia*, whereas bioconcentration in *Chironomus* was approaching a plateau after 72 hours, but did not reach steady state. In *Thymallus* larvae, high uptake persisted even after 168 hours. The bioconcentration factors (BCF) at the end of the exposure periods were 190 ± 50 for *Daphnia*, 680 ± 200 for *Chironomus* and 2200 for *Thymallus*. Of the major metabolites of TPT, only diphenyltin (DPT), but not monophenyltin (MPT) could be detected in the organisms. DPT did not increase significantly in either the organisms or the exposure waters during the experiments. For all species the mortality in the exposure waters did not differ significantly from the control mortality (Table 2-1). The experimentally determined BCF's and toxicokinetic parameters for *Daphnia*, *Chironomus* and *Thymallus* at pH 8.0 and 8.3, respectively, are listed in Table 2-2.

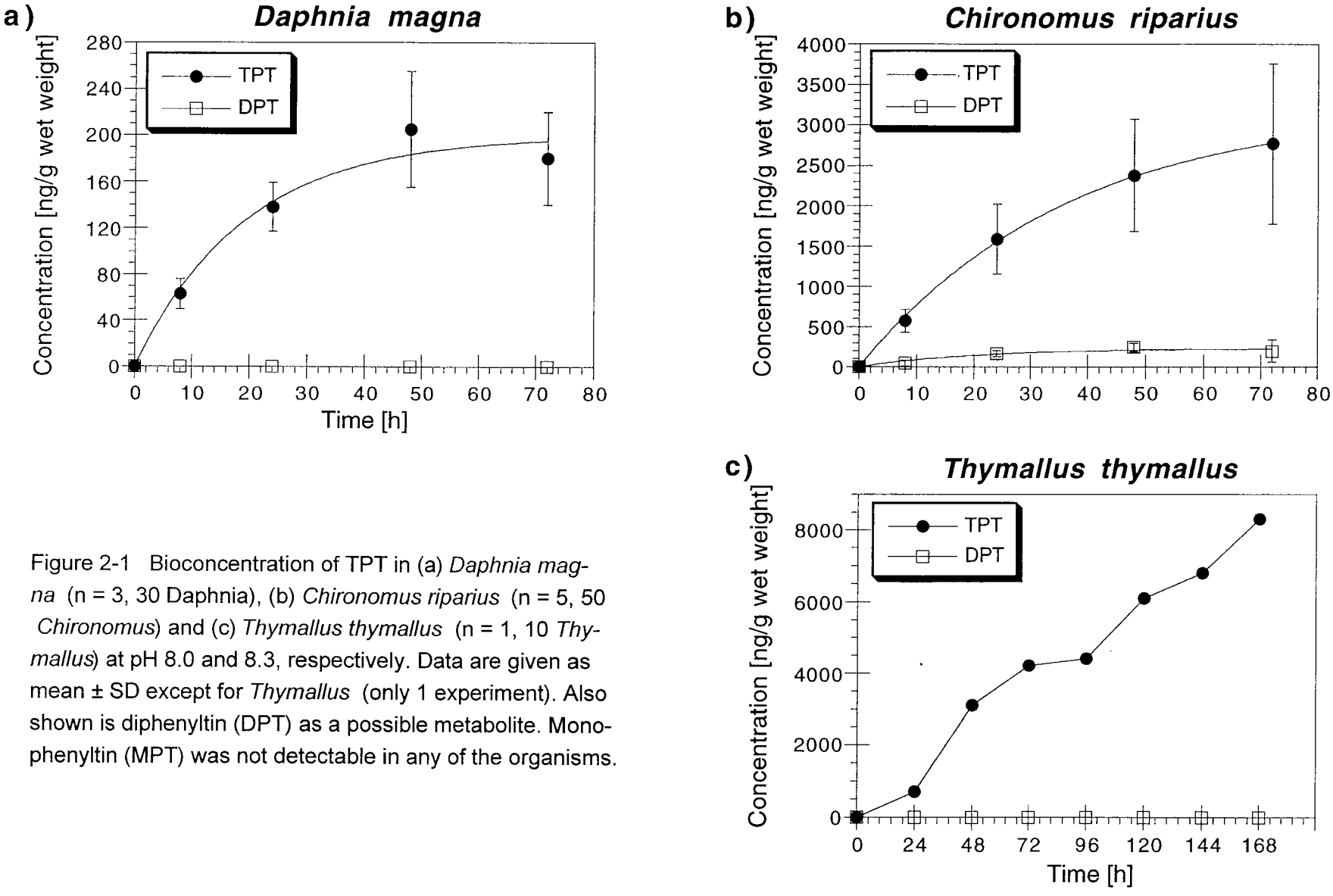

Figure 2-1 Bioconcentration of TPT in (a) *Daphnia magna* (n = 3, 30 Daphnia), (b) *Chironomus riparius* (n = 5, 50 *Chironomus*) and (c) *Thymallus thymallus* (n = 1, 10 *Thymallus*) at pH 8.0 and 8.3, respectively. Data are given as mean ± SD except for *Thymallus* (only 1 experiment). Also shown is diphenyltin (DPT) as a possible metabolite. Monophenyltin (MPT) was not detectable in any of the organisms.

Table 2-2 Toxicokinetic parameters and experimentally determined bioconcentration factors for TPT, TBT and TeBT

Organism	Experiment	Number of replicates	Uptake rate constant k_1 [mL g^{-1} h^{-1}]	Elimination rate constant k_2 [mL g^{-1} h^{-1}]	BCF at end of experiment (c_b/c_w)	Duration of experiment [h]
Daphnia	TPT	3	9.7 ± 2.2	0.049 ± 0.019	190 ± 50	72
Chironomus	TPT	5	19.9 ± 4.0	0.025 ± 0.009	680 ± 200	72
Chironomus	TBT	5	28.6 ± 8.9	0.102 ± 0.039	310 ± 100	72
Chironomus	TeBT	6	17.1 ± 9.5	0.002 ± 0.019	1200 ± 300	72
Thymallus	TPT	1	15.1	0.002	2200	168

Data are given as mean ± SD. The *Daphnia* and *Chironomus* experiments were performed at pH 8.0, the *Thymallus* experiment at pH 8.3. The ionic strength in the experimental water was 13.5 mM for the *Daphnia* and *Chironomus* experiments, and 14.9 mM for the *Thymallus* experiment. A steady state was achieved for TPT in *Daphnia* and for TBT in *Chironomus*. In the TPT experiment with *Chironomus*, bioconcentration was approaching a plateau after 72 hours, but did not reach steady state. In the TeBT experiment with *Chironomus* and in the TPT experiment with *Thymallus*, considerable uptake persisted at the end of the experiment.

Since *Daphnia*, *Chironomus* and *Thymallus* are organisms with different physiological and ecological characteristics, the differences between the BCF's are not surprising. Part of the differences can be explained by the different lipid contents (Table 2-1). On a wet weight base, the total lipid contents for *Daphnia*, *Chironomus* and *Thymallus* are 0.3, 0.6 and 3.1 %, respectively. This results in a ratio of 1 : 2 : 10. The ratio of the determined BCF's in Daphnia, Chironomus and *Thymallus* is 1 : 3.6 : 11.6. It should be noted that in *Chironomus* and *Thymallus* steady state was not reached. Field studies with different vertebrates reported that the distribution pattern of TBT and TPT in the different organs was not related to the lipid content of the corresponding tissues [12, 20]. It can therefore be assumed that species-specific factors other than lipid content such as uptake mechanism and toxicokinetics account for the differences among species.

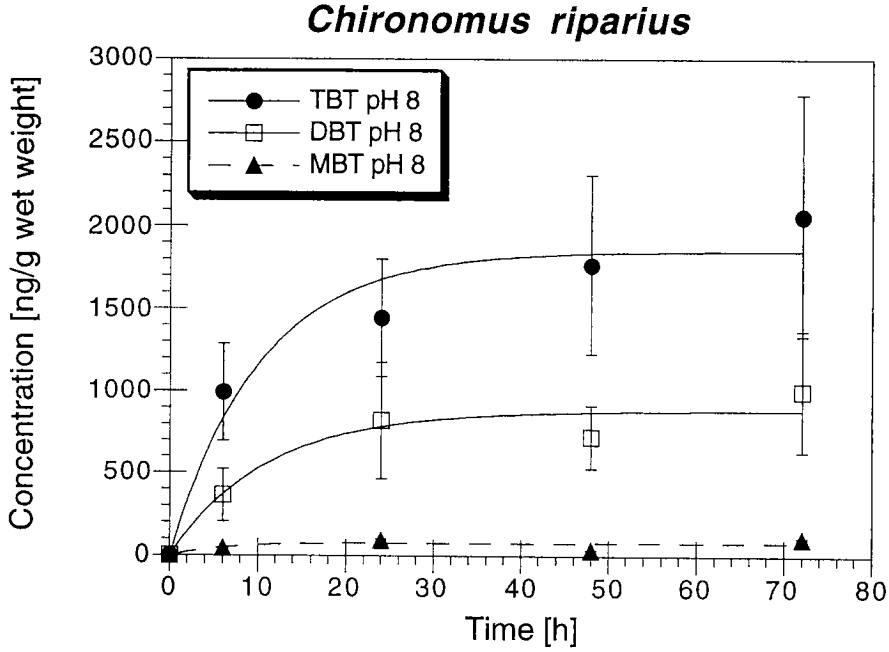

Figure 2-2 Time course of tissue TBT, DBT and MBT concentrations in *Chironomus* larvae. Exposure concentration was 6.5 µg TBT L^{-1} at pH 8.0. Data are given as mean ± SD of 50 *Chironomus* (n = 5). The increasing DBT concentrations indicate metabolism of TBT.

Figure 2-2 shows the bioconcentration of TBT in *C. riparius* at pH 8.0. A steady state was reached within the 72 h exposure period. For TBT, a BCF of 310 ± 100 was determined at the end of the experiment. In contrast to the experiment with TPT, significant concentrations of metabolites were

observed. Dibutyltin (DBT) in the tissues increased during the experiment and reached a steady state (Figure 2). After 72 hours, the DBT tissue residues were about 50 % of the TBT tissue residues. Monobutyltin (MBT) occurred only in low concentrations. The concentrations of DBT and MBT in the exposure waters remained constant over time at levels of 0.38 ± 0.09 μg L^{-1} and 0.12 ± 0.03 μg L^{-1}, respectively, indicating that DBT was not released by the organisms. However, the DBT tissue concentrations leveled off quickly (Figure 2) suggesting that either TBT metabolism slowed down after about 24 hours or the produced DBT was further metabolized.

Based on the log K_{ow} for TPT and TBT at pH 8 (3.5 and 4.1, respectively), a lower BCF would be expected for TPT than for TBT. The determined TPT and TBT bioconcentration factors in *Chironomus* contrast sharply to the predictions of the octanol-water model. The BCF for TBT (310) is more than a factor of two lower than the BCF for TPT (680). This result is related to TBT metabolism in *Chironomus*, whereas no metabolism of TPT could be observed. The presence of an efficient TBT metabolism in *Chironomus* is supported by the fact that the DBT tissue residues increased significantly over time, reaching high levels, while the concentrations of MBT and DBT in the exposure waters remained constant at a low level. Furthermore, achievement of steady state within 3 days is at least partly the result of TBT metabolism, which is indicated by the relatively high elimination rate constant k_2 of 0.10 ± 0.04 h^{-1} (Table 2-2). A short time until steady state (7 days) has also been found for the uptake of TBT by *Hyalella azteca* [43], but concentrations of DBT and MBT were not reported. Stäb et al. [12] found high concentrations of MBT and DBT in chironomids and *Gammarus* and pointed out the possibility of relatively high TBT degradation rates in these organisms. Our study demonstrates TBT metabolism in *Chironomus riparius*, which is of importance for the fate of TBT in the foodwebs of freshwater lakes and rivers as various fish species are predators of chironomids.

A much higher bioconcentration of tetrabutyltin (TeBT) than TPT or TBT was observed in *Chironomus*. The experimentally determined BCF at pH 8.0 was 1200 ± 300 after 72 hours (Table 2-2). No other butyltin compounds occurred at detectable levels in *Chironomus* larvae in this experiment. Metabolism was therefore not present or only of minor importance. To our knowledge, no octanol-water partition coefficient (K_{ow})

for TeBT has been reported so far, but it is expected to be higher than the K_{ow}'s of TPTOH and TBTOH.

2.3.2 Influence of pH

Figure 2-3a shows the bioconcentration of TBT in *Chironomus* larvae at pH 8 and pH 5. A steady state was achieved within the exposure period. The determined BCF's after 72 hours were 310 ± 100 (pH 8) and 170 ± 30 (pH 5). The difference between the BCF's at pH 8 and pH 5 is statistically significant (t-test, two-sided, $p = 0.045$, $n = 5$). As found at pH 8 (Figure 2-2), the DBT residues in the larvae also increased at pH 5 to a level of approximately 50 % of the TBT residues, indicating metabolism of TBT. These findings are consistent with the relatively high elimination rate constants k_2 of 0.10 ± 0.04 h^{-1} (pH 8) and 0.12 ± 0.04 h^{-1} (pH 5) compared to the k_2-values in the TPT experiment (Table 2-2). The bioconcentration of TPT was also higher at pH 8 than at pH 5 (Fig. 2-3b), but the difference was less pronounced than for TBT and was not statistically significant (t-Test, two-sided, $p = 0.17$, $n = 5$). At the end of the experiment, the BCF's were 680 ± 200 (pH 8) and 510 ± 120 (pH 5).

The higher BCF of TBT at pH 8 than at pH 5 may be explained by a higher uptake of the uncharged hydroxide species as compared to the cation. At pH 8 the hydroxide complex TBTOH predominates. The pKa value of TBT is 6.25 [26]. Based on this pKa, the fraction of TBTOH drops below 10 % at pH 5, and the cation TBT^+ becomes the predominant species. TPT has a pKa of 5.2 [26], which results in approximately 40 % TPTOH and 60 % TPT^+ at pH 5. The weaker effect of the pH on the bioconcentration of TPT, compared to TBT, is consistent with the higher fraction of the hydroxide species at pH 5. This indicates that the hydroxide species TBTOH and TPTOH are the predominant form taken up by the organism.

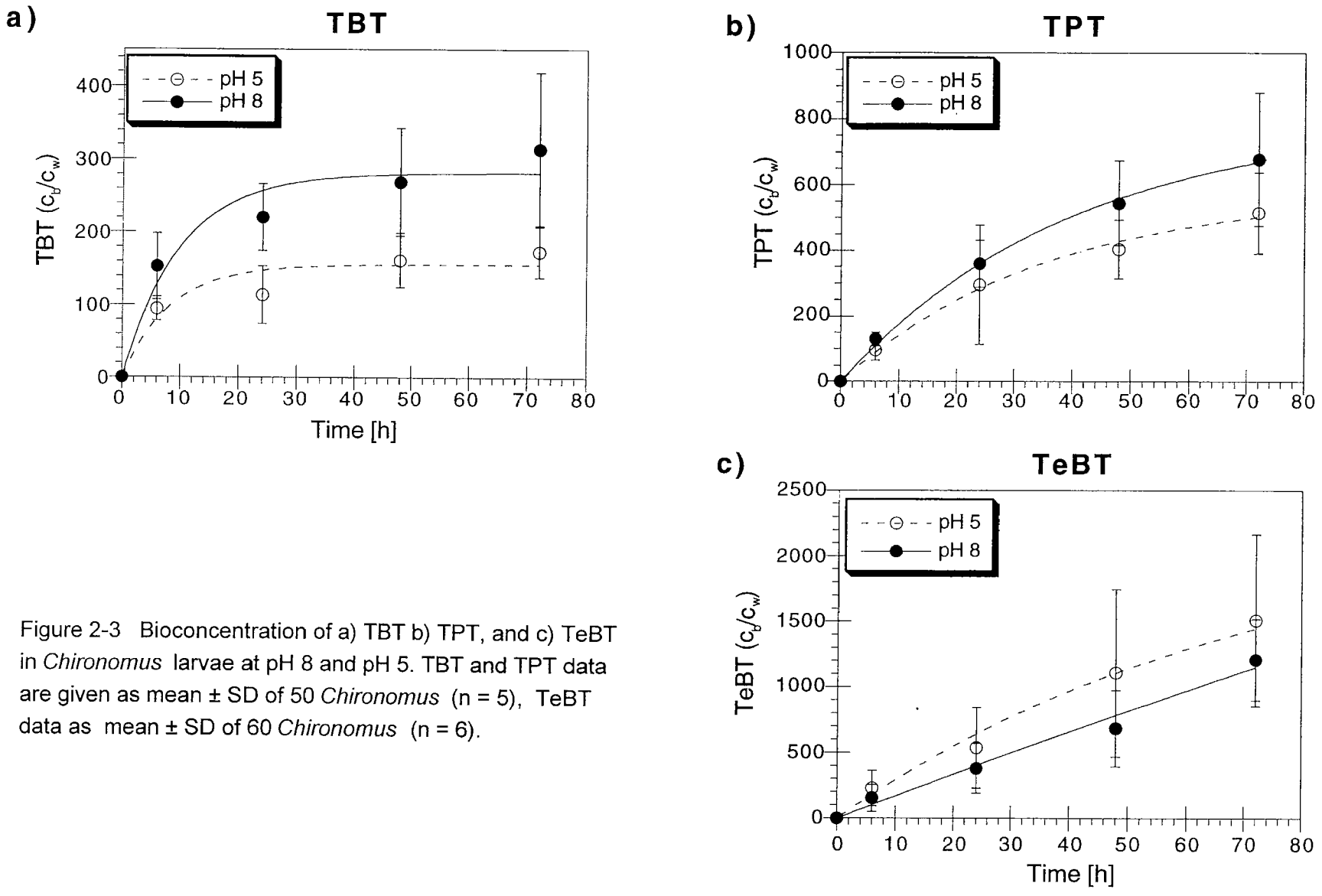

Figure 2-3 Bioconcentration of a) TBT b) TPT, and c) TeBT in *Chironomus* larvae at pH 8 and pH 5. TBT and TPT data are given as mean ± SD of 50 *Chironomus* (n = 5), TeBT data as mean ± SD of 60 *Chironomus* (n = 6).

Although the uptake of TBT and TPT by *Chironomus* larvae was higher at pH 8 than at pH 5, the difference is less pronounced than the octanol-water model would predict. For TBT, the overall octanol-water distribution ratios (log D_{ow}) are 4.1 and 2.9 at pH 8 and pH 5, respectively [26]. Based on these results, a more than tenfold lower accumulation of TBT would be expected at pH 5 than at pH 8. The experimentally determined BCF's (steady state) only differed by a factor of 1.8. Obviously, the octanol-water partition model underestimates the bioconcentration of TBT at pH 5. The discrepancy may be related to different behavior of the TBT^+ cations in biological membranes than in octanol-water systems. It has been shown for substituted phenolic compounds that charged species partition to a higher extent into liposomes than the octanol-water distribution ratios (log D_{ow}) predict [44]. It should be investigated whether or not triorganotin cations behave in a similar fashion as charged species of other hydrophobic ionizable compounds. A further explanation for the discrepancy to the octanol-water model may be pH differences at the uptake membranes as related to the pH of the ambient water. Although the mechanism remains to be elucidated, the results indicate that *Chironomus* larvae take up the cations TBT^+ and TPT^+ more efficiently than the octanol-water partition model suggests.

In the experiment with TeBT (Figure 2-3c), the *Chironomus* larvae showed a slightly higher BCF at pH 5 (1500 ± 650) than at pH 8 (1200 ± 310), but the difference was not significant (t-Test, two-sided, $p = 0.39$, $n = 6$). In contrast to TBT and TPT, TeBT does not dissociate in water. Therefore, no pH-dependence of the TeBT speciation was expected. The similar BCF's for TeBT at pH 8 and pH 5 thus indicate that the H^+ concentration itself has no significant influence on the bioconcentration at these pHs. It can be concluded, therefore, that the differences in the bioconcentration of TBT and TPT at pH 8 and pH 5 are related to the chemical speciation rather than to pH-induced alterations of uptake membranes.

2.3.3 Influence of Humic Substance

Fig. 4a shows the bioconcentration of TPT in *Daphnia magna* in the presence of Aldrich humic substance (HS). The HS concentrations ranged from 0 to 14.2 mg C L^{-1} and the pH was 8.0. For *Daphnia* in exposure waters without HS, a BCF of 190 ± 50 was determined. The BCF's for

experiments with 1.1, 4.4, 8.0 and 14.2 mg C L^{-1} were 150 ± 60, 100 ± 40, 120 ± 40 and 60 ± 30, respectively. The average mortality under TPT exposure (33 ± 12 %, n = 15) was not significantly different from the control mortality (20 ± 11 %, n = 15).

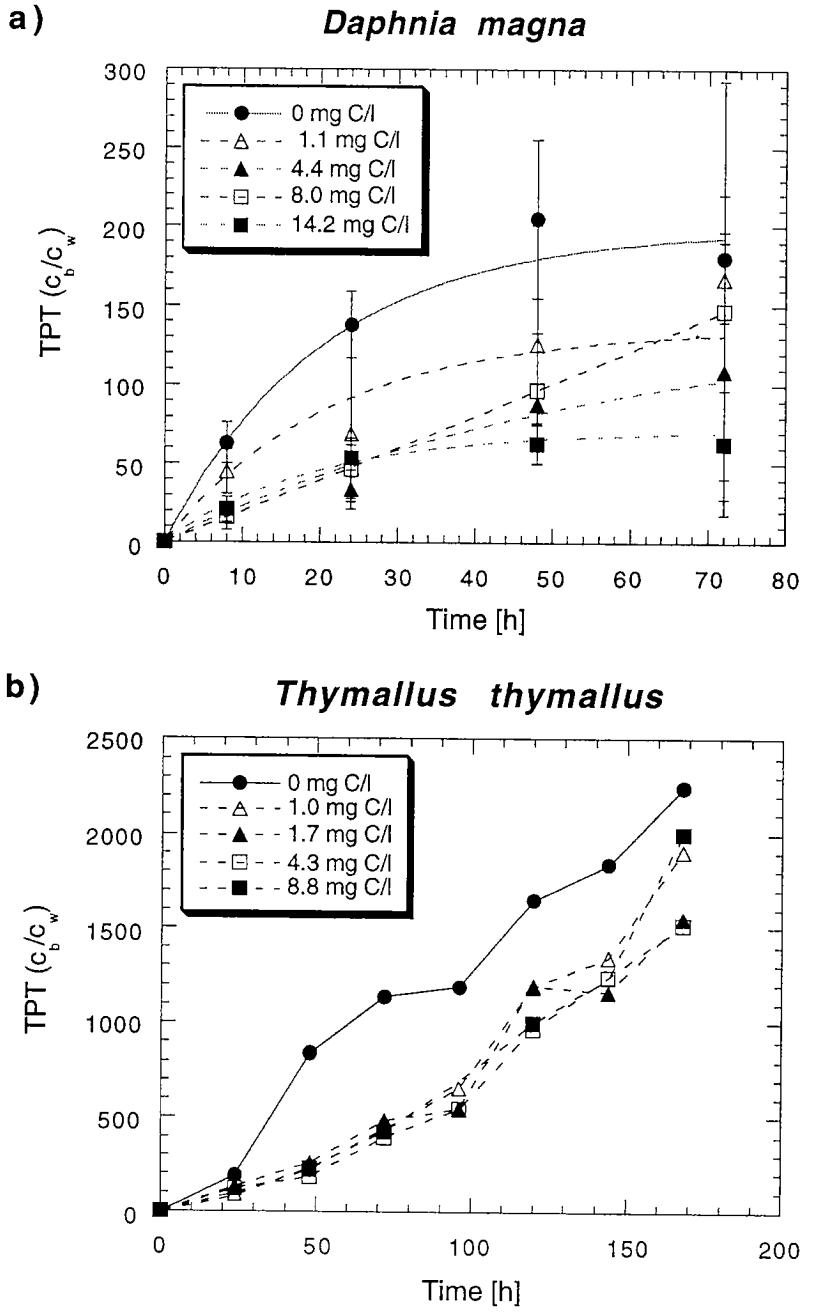

Figure 2-4 Bioconcentration of TPT at different concentrations of humic substances (DOC in mg C L^{-1}) in *Daphnia* (a) and *Thymallus* larvae (b) at pH 8.0 and pH 8.3, respectively. Data given as mean ± standard deviation of 30 *Daphnia* (n = 3) and values for 10 *Thymallus* larvae (n = 1), respectively.

A significant decrease of the BCF was only observable for a HS concentration of 14.2 mg C L^{-1} (t-Test, two-sided, p = 0.03, n=3). Lower DOC concentrations did not produce significant effects. A very similar picture was obtained in experiments with fish yolk sac larvae *Thymallus thymallus* at pH 8.3 (Figure 2-4b). The BCF's at the end of the experiments were 2240 (0 mg C L^{-1}), 1900 (1 mg C L^{-1}), 1550 (1.7 mg C L^{-1}), 1520 (4.3 mg C L^{-1}) and 2000 (8.8 mg C L^{-1}). Although there is a trend towards lower BCF's in the presence of HS, the differences between the determined BCF's are modest and may be attributed to the natural variability of the fish yolk sac larvae. Mean mortality in all tests with TPT (3.5 ± 3.1 %, n = 5) was not significantly different from the control (4 %). Note that steady state was not reached within the 168 hour experimental period.

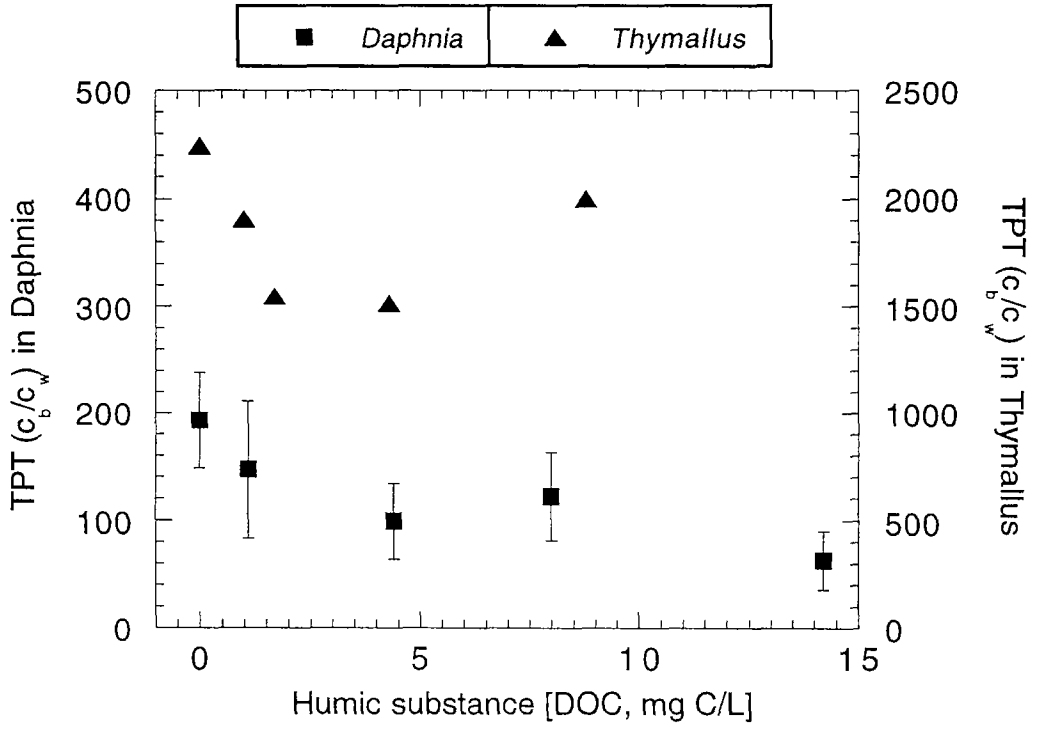

Figure 5 Influence of different concentrations of humic substances (DOC in mg C L^{-1}) on the bioconcentration of TPT in *Daphnia* (a) and *Thymallus* larvae (b). Data refer to the mean bioconcentration factors (c_b/c_w) of 48 and 72 h in *Daphnia* and at the end of the experiment (168 h) in *Thymallus* larvae.

A variety of studies have shown that the bioavailability of hydrophobic organic chemicals is reduced in the presence of humic substances [30-33, 45]. Similar effects were shown for TBT in a previous study with *Daphnia* and *Thymallus* [28]. It can generally be expected that high concentrations of humic substances reduce the bioavailability of organotins. Concentrations higher than 10 mg C L^{-1} are, however, not very representative for many natural freshwater systems. Since the D_{OM}-value of TPT is approximately 15,000 L kg_{OM}^{-1} for Aldrich humic substance (HS) at pH 8 [27], a concentration of 10 mg C L^{-1} is expected to reduce the free TPT concentration by a factor of 1.3. Lower HS concentrations will result in a minor reduction of the bioavailable TPT fraction. The results of the experiments with *Daphnia* and *Thymallus* confirm the hypothesis that relatively high HS concentrations (> 10 mg C L^{-1}) are necessary to generate pronounced effects on the bioavailability of TPT to freshwater organisms (Figure 2-5).

2.4 Conclusions

This study shows differences in the bioaccumulation of TPT in three different freshwater species representing different ecological niches. Total lipid content explains a part of the differences, but other species-specific factors such as uptake mechanism influence the balance between uptake and elimination. Rapid achievement of steady state makes *Chironomus riparius* a promising species for monitoring TBT and TPT availability in contaminated harbor sediments. Furthermore, *Chironomus* is a key organism in benthic communities. Thus, the presence of an efficient TBT metabolism in *Chironomus* larvae can influence the distribution pattern of butyltin compounds in the foodwebs of freshwater lakes. If additional organisms of low trophic levels can metabolise TBT more easily than TPT, a lower biomagnification potential can be expected for TBT than for TPT in general.

Both pH and humic substances influence the bioconcentration of organotins in freshwater organisms. The bioconcentration of TBT and TPT was found to be higher at ambient pH of surface waters than at low pH. The hydroxide species TBTOH and TPTOH appear to be taken up to a somewhat higher degree than the corresponding cations. The cations, however, are

more readily taken up than the octanol-water model would suggest. Humic substances (HS) can reduce the bioavailability of TBT and TPT, but only relatively high concentrations of HS lead to a substantial reduction. In contaminated harbor sediments, however, the bioavailability of TBT or TPT may be reduced by high organic matter contents.

Further experiments with *Chironomus riparius* are needed to answer the open questions concerning organotin mass balances and to provide quantitative toxicokinetic data. Future research should focus on the bioavailability and toxicity of TBT and TPT in sediments.

2.5 Acknowledgements

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Determination of Organotin Compounds in Biota Samples by Cold Methanolic Digestion, Simultaneous Extraction/Derivatization, and GC/MS Analysis

A very efficient extraction procedure for GC analysis of six butyl- and phenyltin compounds in biota samples has been developed. Sample preparation is based on cold digestion in a methanolic solution of acetic acid and sodium acetate with subsequent aqueous ethylation by $NaBEt_4$ and liquid-liquid extraction with hexane. For samples of only 40 mg biological materials, method detection limits ranging from 4 to 52 ng/g were achieved using gas chromatography/mass spectrometry (GC/MS). Relative recoveries for the individual butyl- and phenyltins, referring to perdeuterated organotin analogues as internal standards, ranged from 96 to 107%.

Looser, P.W.; Berg, M.; Fent, K.; Mühlemann, J.; Schwarzenbach, R. P. *Submitted to Anal.*

Organotin concentrations in insect larvae (*Chironomus riparius*) and a reference mussel tissue (CRM 477) were determined with excellent precision (RSD < 5%), and the measured butyltins in CRM 477 were in good agreement with the certified values. Comparison with accelerated solvent extraction (ASE) confirmed the high accuracy, and application for a bioconcentration experiment with phenyltins demonstrated the robustness and suitability of the method for routine analyses. The new procedure allows extremely fast and reliable determination of organotin compounds in low size biological samples.

3.1 Introduction

The environmental relevance and the toxicity of organotin compounds towards aquatic organisms is well documented [1]. Most prominent are tributyltin (TBT) and triphenyltin (TPT), which have been extensively used as biocides in antifouling paints and for crop protection, respectively. Although regulations on the use of TBT-based antifouling paints may have lowered the concentrations in freshwater and marine ecosystems [2-4], contaminated sediments still pose a long-term risk to aquatic wildlife, in particular to burrowing sediment organisms. Sediment-dwellers generally bioaccumulate organotins [5-8] and therefore reflect the bioavailable concentrations in the sediments. Hence, their suitability as sentinels of organotin contamination is obvious, but their low size requires analytical techniques of very high sensitivity.

A variety of different techniques for organotin analysis in solid materials have been applied and critically reviewed [9]. Most of the available methods for biota samples are based on gas chromatography, where derivatization of the organotins is indispensable prior to analysis. This major drawback of the GC techniques can be overcome by simultaneous extraction-derivatization procedures using sodium tetraethylborate ($NaBEt_4$) as ethylating reagent [10-12]. These procedures are much less time-consuming than the traditional alkylation methods with Grignard reagents. However, when dealing with the complex matrices of biota samples, satisfactory derivatization yields can only be obtained, if an appropriate digestion step precedes the extraction-derivatization procedure.

Recently, methanolic solutions of acetic acid have shown to be very efficient for extracting organotins from sediments under microwave-assistance [13] or with accelerated solvent extraction (ASE) [12]. Thereby, the acetic acid acts as complexing agent, which results in enhanced extraction recoveries [12]. For biological materials, methanol is an efficient tissue digester, since it may enter cells of organisms very rapidly so that they burst within a few seconds. Consequently, organisms of small size can easily be digested without tissue solubilizers such as TMAH, KOH or enzymes [9]. The result is a very homogenous suspension that can directly be transferred into an aqueous solution for derivatization and liquid-liquid extraction [14].

With the new method presented in this paper, butyl- and phenyltin compounds in biological materials can be determined extremely fast and with high accuracy and precision by GC/MS. Our experience shows that compared to traditional Grignard methods, much higher numbers of samples can be processed per time. In contrast to other methods [9], no clean up step is necessary for sample sizes up to 500 mg, and a good performance of the GC/MS systems can be maintained for high numbers of subsequent injections. The validation of the method included the direct comparison with an ASE method [12], determination of recovery rates of six individual butyl- and phenyltins in spiked samples, and analysis of the certified reference mussel tissue CRM 477 [15]. Application of the method for phenyltin-exposed insect larvae demonstrates the efficiency and suitability of the method for routine analyses.

3.2 Experimental Section

3.2.1 Materials

Tributyltin chloride TBTCl (> 97%), dibutyltin dichloride $DBTCl_2$ (~ 97%), triphenyltin chloride TPTCl (> 97%), tetrabutyltin TeBT (≥ 98%), and acetic acid (> 99.5%) were obtained from Fluka Chemie AG (Buchs, Switzerland). Diphenyltin dichloride $DPTCl_2$ (> 98%) was purchased from ABS (Basel, Switzerland). Butyltin trichloride $MBTCl_3$ (~ 95%) and phenyltin trichloride $MPTCl_3$ (~ 98%) were obtained from Aldrich, Steinheim (Germany). Ethylated butyl- and phenyltin compounds (MBT-Et_3,

DBT-Et_2, TBT-Et, MPT-Et_3, DPT-Et_2, TPT-Et) and perdeuterated organotin compounds (MBT-d_9, DBT-d_{18}, TBT-d_{27}, MPT-d_5, DPT-d_{10}, TPT-d_{15}; as respective chlorides) were synthesized in our laboratories.[16] Sodium tetraethylborate ($NaBEt_4$; > 98%) was obtained from Strem Chemicals (Bischheim, France). Methanol (> 99.9%) was purchased from Scharlau (Barcelona, Spain). Sodium acetate (> 99%), sodium chloride (> 99%), sodium hydroxide (> 99%), hydrochloric acid (32% p.a.), nitric acid (65% p.a.), and hexane (> 99%) were from Merck (Darmstadt, Germany). Nanopure water was obtained from deionized water which was further purified with a Nanopure water purification device (NANOpure 4, Skan, Basel, Switzerland).

All reported organotin concentrations in this paper refer to Sn. Organotin chlorides (70–140 mg) were dissolved in 50 mL methanol containing 0.01 M HCl (1.1 mL HCl 32% in 1000 mL methanol). Ethylated standards and tetrabutyltin solutions were prepared in hexane. All standards were kept at 4 °C. The primary standards were renewed after six and the dilutions after three months. In previous work, it was found that solutions were stable for more than six months [12]. Aqueous solutions of $NaBEt_4$ (2% w/v) were prepared daily. All glassware was rinsed with methanol (0.01 M HCl), soaked overnight in 1 M HNO_3 to remove sorbed organotin compounds, and rinsed again with Nanopure water. ASE extraction cells were dismantled, thoroughly rinsed with tap water, and sonicated twice in methanol for 30 min.

Beware: *Organotin compounds are toxic. Skin contact or inhalation of vapors must be avoided. Pure $NaBEt_4$ is self flammable, and water for preparing the solutions should be ready before weighing, in order to minimize the contact time of the $NaBEt_4$ with air.*

Chironomus riparius larvae were cultured in our laboratories [17] and exposed to organotin compounds for comparing cold methanolic digestion with ASE, for the determination of method detection limits (MDL), and for studying the bioconcentration of phenyltin compounds. The exposure was performed in a climate chamber at 20 ± 2 °C with a 16:8 hours light:dark cycle. This experimental setup is described in Chapter 2. The reference mussel tissue CRM 477 with a certified content of butyltin compounds [15] was obtained from the Institute for Reference Materials and Measurements (IRMM) of the European Commission (Geel, Belgium) and stored at -25 °C until analysis.

3.2.2 Cold Methanolic Digestion of Biological Material

The scheme in Figure 3–1 gives an overview of the cold methanolic digestion procedure. The fresh, frozen or lyophilized biological materials were weighed and transferred into conic 10 mL glass centrifuge tubes (NS 14/23). A solution of 1 M sodium acetate and 1 M acetic acid in methanol was added to achieve a total volume of 3 mL. The materials were homogenized (4 min) with a 3 mm diameter glass stick and spiked with 50 µL perdeuterated internal standard mixture (1.0 ng/µL in methanol). The centrifuge tubes were sealed with glass stoppers and shaken briefly. While gently shaking the tubes in for two hours on a horizontal shaker at 150 rpm, the internal standards were allowed to equilibrate between the particles of the suspension and the liquid phase in order to provide optimum imitation of the analytes.

3.2.3 Derivatization with NaBEt4, and Liquid-Liquid Extraction with Hexane

Aqueous Ethylation with $NaBEt_4$ and simultaneous extraction with hexane was performed in 100 mL volumetric flasks („derivatization reactors") which were filled with 2.9 g NaCl, 20 mL Nanopure water and 1.6 mL 2 M NaOH (step 4 in Figure 3–1). The digested homogenates were quantitatively transferred from the centrifuge tubes into the volumetric flasks, followed by rinsing them twice with 2 mL of methanol (1 M NaAc/1 M HAc). The volumes were completed to 100 mL with Nanopure water, the flasks shaken briefly and the pH was adjusted to 5.0 ± 0.1. Finally, 1 mL of freshly prepared aqueous $NaBEt_4$ solution 2% (w/v) and 1 mL of hexane were added, the flasks were sealed with glass stoppers and shaken over night at 200 rpm. If the hexane/water phase separation was not satisfactory, the extracts were centrifuged in 4 mL glass vials at 5000 rpm for 15 min. Aliquots of 500 µL of the hexane extracts were transferred into 1-mL autosampler vials and spiked with 5 µL of the surrogate standard TeBT (20 ng/µL in hexane; for monitoring the GC/MS performance). The hexane extracts were analyzed within few hours or stored at -25 °C, if a delay occurred.

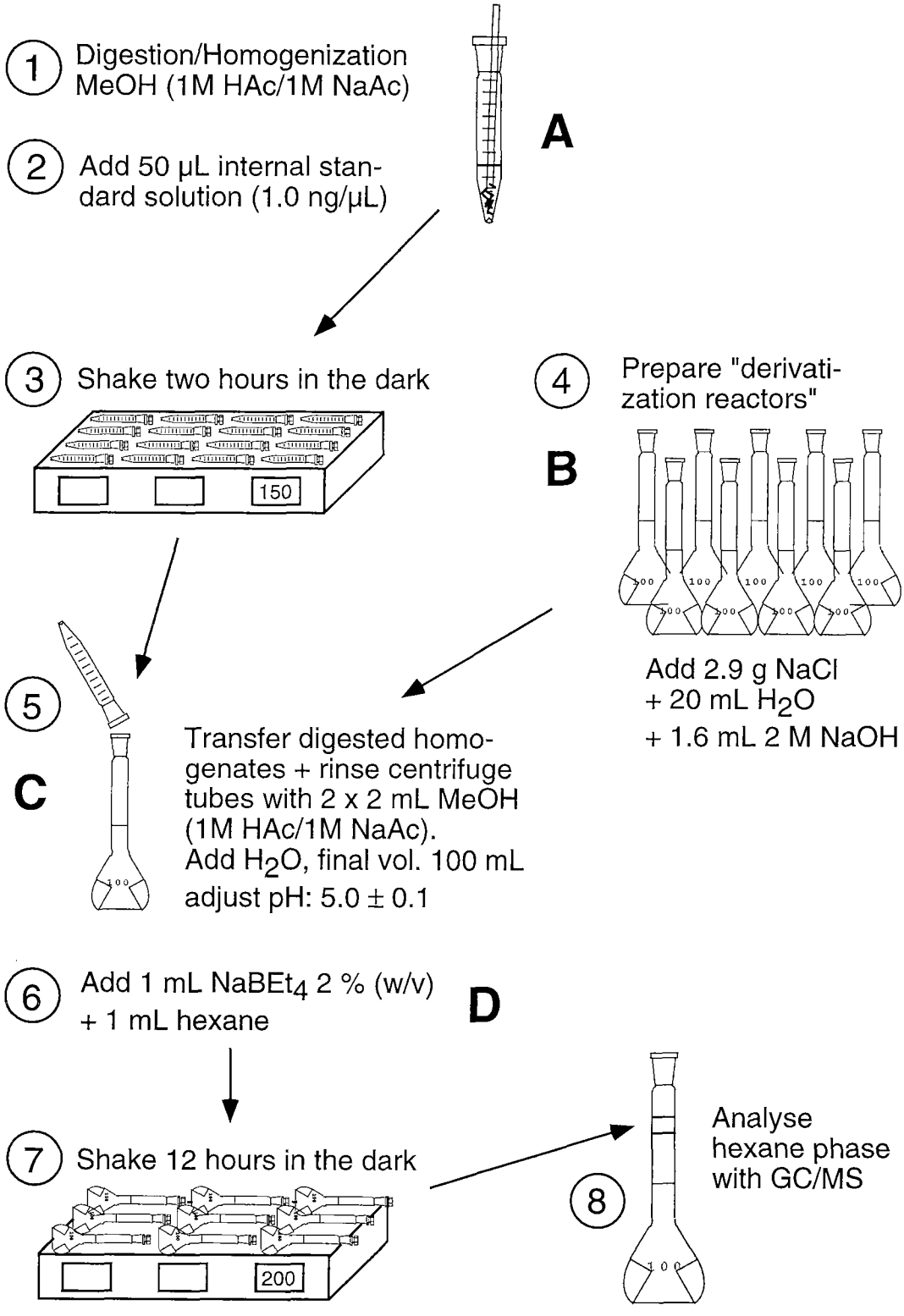

Figure 3–1. Scheme of the sample preparation procedure with cold methanolic digestion. Required time per sample A: 5–7 min, B: 3–5 min, C: 5–7 min, D: 2 min. Up to 24 biota samples can be processed from step one to step six by one person within one single working day.

3.2.4 Calibration Standards

Aqueous standard solutions were used to obtain calibration curves. Accordingly, 100 mL flasks were filled with 2.9 g NaCl, 20 mL Nanopure water, 1.6 mL 2 M NaOH, and 7 mL of methanol (1 M NaAc/1 M HAc). Then, 20, 40, 60, 80, 100 µL organotin chloride standard mixture (1.0 ng/µL in methanol) and 50 µL perdeuterated internal standard mixture (1.0 ng/µL in methanol) were spiked to the aqueous solutions and the flasks shaken briefly. The volumes were completed to 100 mL with Nanopure water and the aqueous standards were derivatized/extracted as described above.

3.2.5 Sample Extraction with Accelerated Solvent Extraction (ASE)

Approximately 500 mg reference material CRM 477 were weighed into 25 mL glass beakers and homogenously spiked with 200 µL perdeuterated internal standard mixture (1 ng/µL in methanol). 1 g quartz sand (Merck) was added and thoroughly mixed with the reference material. The mixtures were filled into 2 mL extraction cells, which were completed with quartz sand. After two hours contact time, they were extracted with the Dionex ASE 200 apparatus, using a solution of 1 M sodium acetate and 1 M acetic acid in methanol as extraction solvent. The extraction procedure included five static extraction cycles of 5 min at 100 °C and 103 bar which is described in detail elsewhere [12]. The combined ASE extracts (7–10 mL) were transferred to 100 mL volumetric flasks, and the derivatization and liquid-liquid extraction were accomplished as described for the cold methanolic digestion homogenates.

For extracting *Chironomus* samples with ASE, the larvae were homogenized in a glass homogenizer and spiked with 400 µL perdeuterated internal standard mixture (1 ng/µL in methanol). Aliquots (250 µL) of the slurry were then directly introduced into the 2 mL extraction cells. The volume was completed with 1 g quartz sand, and the accelerated solvent extraction, derivatization and liquid-liquid extraction performed as described above.

Table 3–1. Determined Compounds, Retention Times, and Mass Traces Monitored [a]

Compound	Retention time [min]	Target ion [m/z]	Qualifier ion [m/z]
MBT-d_9	4.9	244	242
MBT	4.9	235	233
DBT-d_{18}	6.9	279	281
DBT	7.1	263	261
TBT-d_{27}	8.8	217	215
TBT	9.0	291	289
TeBT	10.7	231	233
MPT-d_5	8.2	260	258
MPT	8.2	253	251
DPT-d_{10}	13.1	313	311
DPT	13.2	303	301
TPT-d_{15}	17.3	366	364
TPT	17.4	351	349

[a] Single ion recording (SIR) in seven retention windows as follows: 4.3–5.5; 6.5–7.7; 7.7–8.6; 8.6–9.4;10.0–11.5; 12.0–14.5; 16.0–18.0 min.

3.2.6 Gas Chromatography/Mass Spectrometry (GC/MS)

The hexane extracts were analyzed with a Fisons GC 8060 gas chromatograph coupled with a Fisons MD 800 mass spectrometer. 1 µL extract was injected on column. The GC was equipped with a 1.2 m x 0.53 mm deactivated fused-silica precolumn and a 15 m x 0.25 mm capillary column DB-5 (film thickness 0.25 µm). The helium flow was regulated in the pressure control mode (150 kPa). The GC temperature programm was 1 min at 60 °C, to 250 °C at 10 °C /min, and 5 min at 250 °C. The GC/MS interface had a temperature of 250 °C. Detection was performed in the electron impact (EI+) mode and with single ion recording (SIR) using the target and qualifier ions listed in Table 3–1. The linear range of the GC/MS system is specified in ref. [12]. Figure 3–2 shows a typical SIR chromatogram obtained from a hexane extract of 11 *Chironomus* larvae (40 mg) containing MBT, DBT, TBT, MPT, DPT, and TPT. High resolution

and good peak symmetry was achieved for up to 48 subsequent injections of biota extracts.

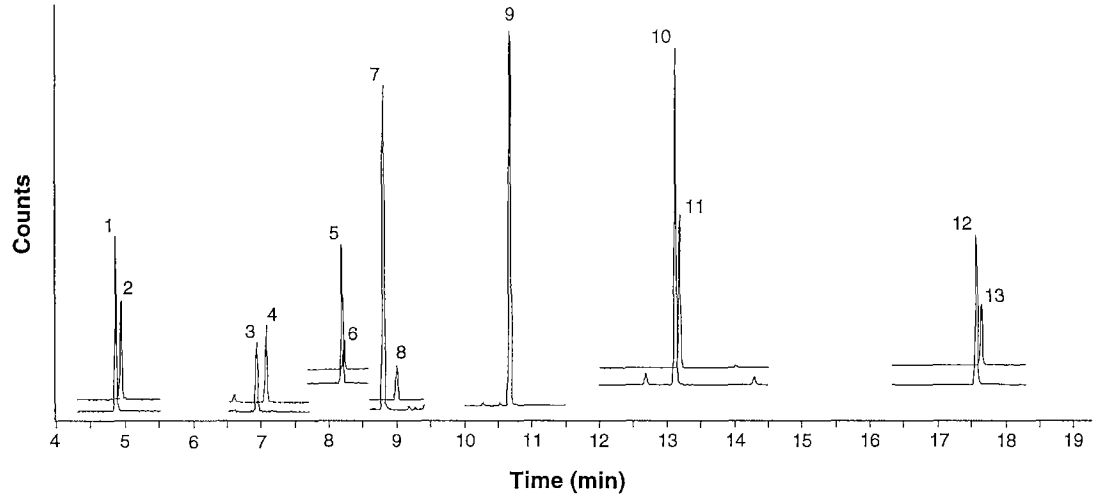

Figure 3–2. SIR Chromatogram (mass traces of the quantification ions) of derivatized organotin compounds in a 40 mg *Chironomus* larvae extract. The concentrations in the larvae were 0.53, 0.75, 0.69, 0.76, 0.61, and 0.36 µgSn/g wet weight for MBT, DBT, TBT, MPT, DPT, and TPT, respectively. TeBT was used as surrogate standard. Peak identifications: (1) MBT-d9, (2) MBT, (3) DBT-d18, (4) DBT, (5) MPT-d5, (6) MPT, (7) TBT-d27, (8) TBT, (9) TeBT, (10) DPT-d10, (11) DPT, (12) TPT-d15, (13) TPT.

Quantification was generally accomplished by internal standardization using perdeuterated organotin compounds as internal standards, with the exception of the absolute recovery rates (see next section).

3.2.7 Determination of Absolute and Relative Recoveries and Method Detection Limits (MDL)

The recovery rates were determined with unexposed, but spiked *Chironomus* larvae. Absolute recoveries were determined to obtain information about extraction and derivatization yields, whereas the reliability of the method was checked with the relative recoveries. Since degradation of tri- and diorganotin compounds typically results in di- and monoorganotins, recovery rates are only meaningful, when determined for individual butyl- and phenyltins. Hence, the absolute and relative recoveries

were determined individually for each compound by separately spiking mono-, di-, and triorganotin chlorides to different samples of homogenized *Chironomus* larvae. The samples for relative recoveries were additionally spiked with perdeuterated organotin compounds as internal standards. After spiking, the samples were set aside for a contact time of four hours, extracted, derivatized, and analyzed as described above. For absolute recoveries, the GC/MS system was calibrated with ethylated organotin standards dissolved in hexane, and using TeBT as surrogate standard. Relative recoveries were obtained from internal calibrations. The method detection limit (MDL) was defined as three times the standard deviation of low concentration levels [18], and determined in unspiked *Chironomus* larvae after exposure to organotin compounds.

3.2.8 Bioconcentration Experiment

A bioconcentration experiment with phenyltin compounds was performed to check the capacity and suitability of the new procedure for studies with high numbers of biota samples. Three replicates of 80 larvae were exposed each in 800 mL solution containing a mixture of $MPTCl_3$, $DPTCl_2$, and TPTCl. The initial concentrations in the 1 L glass beakers were 4.1 ± 0.4 µg Sn/L $MPTCl_3$, 3.7 ± 0.1 µg Sn/L $DPTCl_2$, and 0.8 ± 0.1 µg Sn/L TPTCl. Three further replicates of 30 larvae were exposed to 400 mL solution that contained exclusively $MPTCl_3$ (3.6 ± 0.8 µg Sn/L), and three replicates of 30 larvae only to 3.0 ± 0.1 µg Sn/L $DPTCl_2$. The pH was measured in four hours intervals up to 12 hours of exposure and then twice a day, and ranged from 7.3 to 7.9. After 4, 8, 12, 24, 48 and 72 hours, samples of 10 *Chironomus* larvae were removed, rinsed, dried on filter paper, weighed into vials, and stored at -20 °C until sample preparation. Simultaneously, water samples (25 mL) were decanted, stored at 4 °C and processed within less than 24 hours by aqueous derivatization with $NaBEt_4$ and liquid-liquid extraction with hexane, following the procedure described in ref. [12].

3.3 Results and Discussion

3.3.1 Cold Methanolic Digestion

The function of the extraction solvent is to digest the sample matrix as far as possible and to remove the analytes from the binding sites of the solid material e.g. by complexation of organotin species. In this study, the first task was fulfilled with methanol, whose osmotic acitivity induces rapid bursting of the cells and facilitates homogenisation of the biological material. The acetic acid in the methanolic solution (1 M) acted as complexing agent [12, 13]. In the case of sediments, the digestion and ligand exchange processes have to be enhanced by microwave fields [13], high temperature and pressure [12], sonication [19], or supercritically fluid CO_2 [20]. Biological tissues offer the advantage of better solubility in solvents, particularly if the samples are of small size. This allows digestion and extraction of the organotins at ambient temperature with satisfactory recovery rates, as is demonstrated for the spiked *Chironomus* larvae samples in Table 3–2. The relatively mild extraction method resulted in absolute recoveries of 56–72%. However, the relative recoveries for all six compounds were 96–107%. These results demonstrate, that the perdeuterated internal standards provide an excellent reflection of the analytes behavior and that the advantage of rapidity is definitely not related to a lowered reliability. As can be seen in Table 3–2, the method detection limits (MDL) ranged from 4 to 52 ng Sn/g wet weight. Note that these MDL refer to a sample size of only 40 mg (11 *Chironomus* larvae), and much lower MDL can be obtained with sample sizes of 250–500 mg. Efficient tissue digestion is the prerequisite for a highly homogenous suspension and good reproducibility. As shown in Table 3–2, the relative standard deviations (RSD) of 0.3–2.3%, resulting from extremely small samples of only 40 mg, demonstrate that reproducibility was high with the applied digesting reagent. Furthermore, this excellent precision indicates that the variability of the recovery rates is mainly related to the procedure of spiking organotin chlorides into the biological material and not to the sample preparation procedure.

Table 3–2. Recoveries, Method Detection Limits (MDL), and Relative Standard Deviations (RSD) Determined for Midge Larvae *Chironomus riparius* Using Cold Methanolic Digestion

Compound	90–270 mg *Chironomus* larvae (n=6) [a]			40 mg *Chironomus* larvae (n=5) [b]	
	Spike concentration (ng Sn/g wet w.)	Absolute recovery [c] (%) (RSD)	Relative recovery [d] (%) (RSD)	MDL [e] (ng Sn/g wet w.)	RSD (%)
MBT	407 ± 81	56 (32)	107 (10)	10	0.6
DBT	517 ± 161	72 (21)	96 (7)	20	0.9
TBT	554 ± 75	67 (15)	106 (14)	31	1.5
MPT	521 ± 30	62 (32)	99 (9)	52	2.3
DPT	517 ± 161	[f]	96 (3)	38	2.1
TPT	554 ± 75 [g]	56 (4) [g]	104 (19) [g]	4	0.3

[a] Experiments were performed in six replicates separately for every single compound. [b] Samples exposed to an aqueous mixture of all compounds. [c] Determined in spiked larvae by external calibration with ethylated organotins. [d] Determined in spiked larvae by internal calibration with perdeuterated organotins as internal standards. [e] MDL defined as three times the standard deviation of low concentrations [18]. [f] Not determined. [g] Three replicates.

3.3.2 Comparison of Cold Methanolic Digestion (CMD) with ASE

Figure 3–3 illustrates the results of comparative organotin concentrations measured by CMD and ASE in sample aliquots from 180 *Chironomus* larvae that were all exposed in the same glass beaker. With the CMD method, a somewhat lower MBT concentration was determined than with ASE. Considering the variations of measured MBT concentrations that are reported in ref. [15] (19% RSD after discarding three outlier results), the difference observed here is acceptable. For DBT and TBT, the agreement between both methods was excellent. For all three phenyltin compounds, the agreement between both methods was very good in view of the variability of phenyltin measurements with different methods [21]. The results in Figure 3–3 demonstrate, that with CMD, equal accuracy as with costly extraction techniques such as ASE can be obtained.

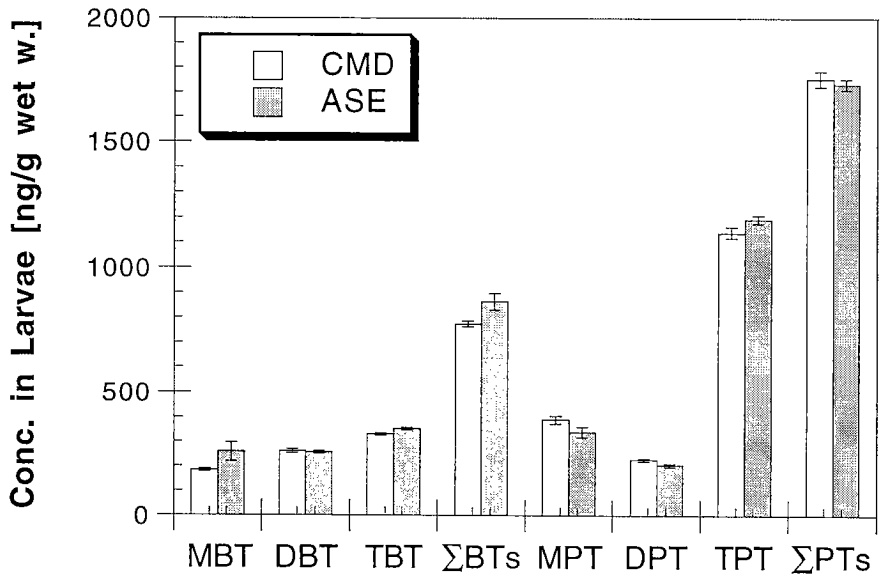

Figure 3–3. Comparison of cold methanolic digestion (CMD) with accelerated solvent extraction (ASE) for Chironomus samples. For both methods, 4 sample aliquots were prepared from 180 larvae, which had been exposed in the same glass beaker to butyl- and phenyltins for 66 h. Data are given as mean ± SD (n = 4).

3.3.3 Analysis of Reference Material CRM 477 (Mussel Tissue)

The CRM 477 was prepared from mussels containing high organotin concentrations, which were collected 1991 in the La Spezia Gulf, Italy [15]. The material is certified for MBT, DBT and TBT and indicative values with high uncertainties have been reported for MPT, DPT and TPT [21]. The concentrations measured with the cold methanolic digestion method as well as by ASE are listed in Table 3–3. CMD yielded somewhat higher MBT concentrations than the certified value. For DBT, excellent agreement was achieved. The measured TBT concentration exceeded the certified value slightly, but was in good agreement with the value determined by ASE. The material is not certified for phenyltins, but the determined concentrations may serve as indicative values. Slightly higher precision was achieved with ASE than with CMD. Nevertheless, the precision of the cold methanolic digestion method was very high (RSD < 5%) for all butyltins and the CRM 477 analyses confirm the accuracy as well as the precision of this new and fast technique.

Table 3–3. Reported and Measured Butyltin and Phenyltin Concentrations in Reference Material CRM 477 (Mussel Tissue)

Compound	Reported values (µg Sn/ g dry w.)	Extraction by ASE [c] (µg Sn/ g dry w.)	Cold methanolic digestion [d] (µg Sn/ g dry w.)
MBT	1.01 ± 0.19 [a]	1.05 ± 0.01	1.22 ± 0.02
DBT	0.79 ± 0.06 [a]	0.79 ± 0.02	0.75 ± 0.03
TBT	0.90 ± 0.08 [a]	1.02 ± 0.01	1.07 ± 0.05
MPT	0.54 ± 0.28 [b]	0.49 ± 0.03	0.48 ± 0.06
DPT	0.06 ± 0.03 [b]	0.06 ± 0.01	0.05 ± 0.01
TPT	0.54 ± 0.14 [b]	0.66 ± 0.01	0.51 ± 0.01

[a] Certified value [15]. [b] Indicative value, from ref. [21]. [c] Mean ± SD (n = 3), sample sizes 505–517 mg. [d] Mean ± SD (n = 6), sample sizes 501–606 mg.

3.3.4 Application to Phenyltin-Exposed *Chironomus* Larvae

In a recent study, MPT, DPT, and TPT concentrations in fish from a pond near TPT sprayed pecan trees were reported [22]. Interestingly, MPT was the predominant organotin species in all fish livers. It was hypothesized that the source of MPT were pecan leaves in the water rather than TPT metabolism in the fish liver. This implies the question, to what extent the phenyltin species MPT and DPT are taken up by freshwater organisms, as compared to TPT. We exposed *Chironomus riparius* larvae to aqueous solutions of $MPTCl_3$ and $DPTCl_2$ separately as well as to mixtures of $MPTCl_3$, $DPTCl_2$, and TPTCl. The larvae were analyzed with the new CMD method and the aqueous phenyltin concentrations were determined as described elsewhere [12].

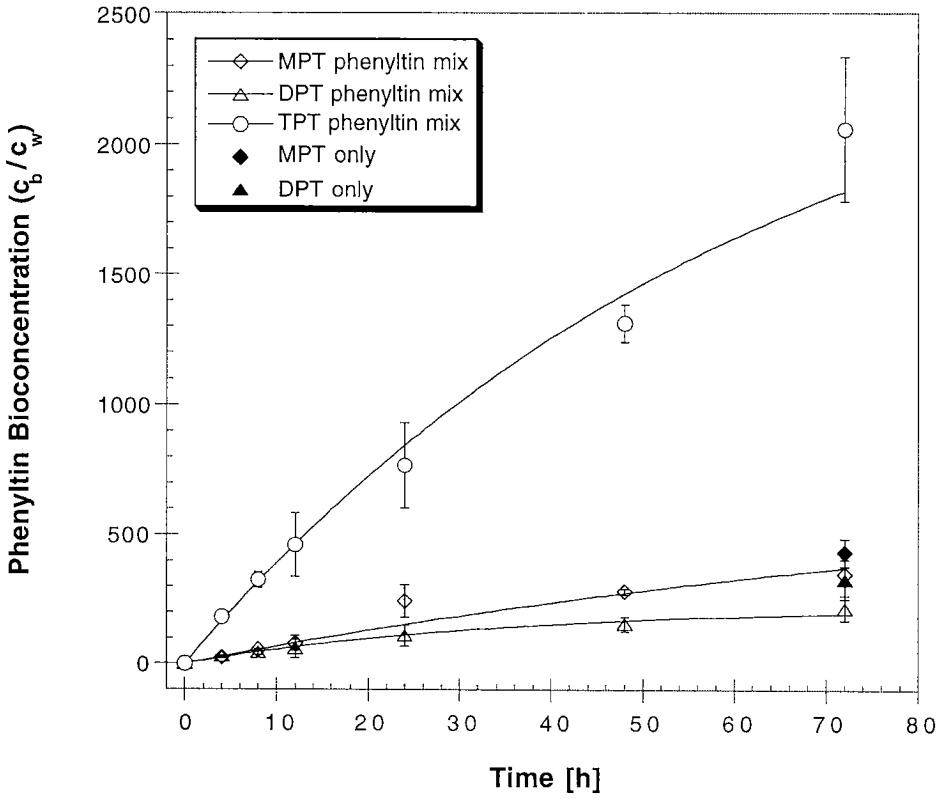

Figure 3–4. Time-course of MPT, DPT, and TPT bioconcentration in *Chironomus riparius* larvae, exposed to mixtures of all three phenyltins (open markers) and to MPT and DPT only (filled markers). The measured concentrations in the larvae (c_b) were divided by the measured concentrations in the water (c_w). The data are given as mean ± SD (n = 3). The solid lines represent the bioconcentration calculated with the model described in Chapter 2.

Figure 3–4 illustrates the time-course of phenyltin uptake by *Chironomus* larvae, expressed as bioconcentration factors, i.e., the concentrations in the larvae divided by the concentrations in the water. MPT and DPT bioconcentration reached much lower values than TPT, but the MPT bioconcentration factor at 72 hours was still higher than 300, which demonstrates the relevance of MPT uptake in a representative freshwater organism. The DPT bioconcentration factor at 72 hours resulting from DPT-only exposure (filled triangle in Figure 3–4) was similar to the respective value obtained in the mixture. DPT was therefore taken up from water and did hardly originate from TPT metabolism. This confirms the results of a previous study, in which very slow TPT metabolism in *Chironomus riparius* was suggested (Chapter 2). Note that all 24 biota samples of the experiment were processed by one person within one single working day and although no clean up step was provided, a good performance of the GC/MS system was maintained during 48 subsequent injections of biota extracts. This demonstrates the high efficiency of the method and its suitability for routine analysis.

3.4 Conclusions

With the described new method, organotin concentrations in biota samples of small size can be determined with high accuracy and precision. The methanolic solution of acetic acid and sodium acetate provides very efficient digestion of the sample matrix without additional tissue solubilizers and without costly instrumentation. The efficiency of this cold methanolic digestion, and the fact that no cleanup step is necessary, generate a very fast procedure, which allows to process high numbers of samples within extremely short time. Hence, the method offers a rapid screening tool of high reliability for organotin-exposed organisms. The method development and validation was based on GC/MS and perdeuterated organotin compounds as internal standards. However, with tripropyl- and tripentyltin as internal standards, the hexane extracts obtained with the described procedure can also be analyzed with the less expensive gas chromatography/flame photometric detection (GC/FPD) technique.

3.5 Acknowledgements

The authors thank Alfredo C. Alder and Torsten C. Schmidt for reading the manuscript.

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Uptake and Elimination of Triorganotin Compounds by Larval Midge *Chironomus riparius* in the Absence and Presence of Aldrich Humic Acid (AHA)

Sediments contaminated with triorganotin compounds (TOTs) are abundant in areas with high shipping activities. In such areas, sediment-dwellers such as chironomids may introduce these very toxic contaminants into foodwebs. In this work, we studied systematically the uptake and elimination of tributyltin (TBT) and triphenyltin (TPT) with larvae of *Chironomus riparius* at pH 5 and pH 8, and in the absence and presence of Aldrich humic acid (AHA). Both uptake and elimination data could be simultaneously fit by a two-pool model, and uptake, excretion and metabolism rate constants could be derived with very good reproducibility.

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Bioconcentration was found to be significantly lower for TBT than for TPT, although TBT is more hydrophobic. The major reason was the rapid metabolism of TBT to dibutyltin (DBT) and further to monobutyltin (MBT). Apparent AHA-water distribution ratios (D_{AHA}) were derived from bioconcentration experiments in the presence of AHA and were compared with D_{AHA} values determined in dialysis experiments. Except for some explainable discrepancies, a good agreement between both sets of D_{AHA} values was found, confirming the hypothesis that TOTs associated with AHA are not bioavailable. The results of this work demonstrate that chironomids are well suited organisms to study the speciation and bioconcentration of organotin compounds present in sediment pore waters.

4.1 Introduction

Triorganotin compounds (TOTs), in particular tributyltin (TBT) and triphenyltin (TPT) are recognized as highly toxic to aquatic organisms and a wide variety of detrimental effects have been reported [1]. Restrictions in the use of TBT as antifouling agent in boat paints have resulted in lower concentrations in freshwater marinas and coastal marine ecosystems [2-4]. However, despite these measures, high TBT concentrations may persist for years in sediments [5-12], where benthic organisms are directly exposed to the contaminant. TPT has been applied as co-toxicant in some antifouling paints, but is mainly used as fungicide in agriculture [13, 14], where no restrictions on its use have been established to date.

Although the potential of sediment-dwellers for TOT uptake and subsequent transfer into food chains is obvious, little is known about the fate in representative freshwater sediment organisms. The relatively high hydrophobicity of neutral TBT and TPT species (at pH 8, log K_{OW} are 4.1 and 3.5, respectively [15]) as well as the ability of TOT cations to form strong complexes with oxygen ligands [15] suggest a high bioconcentration potential, which is confirmed by laboratory and field studies [1]. The actual degree of bioconcentration is, however, not only a question of the compounds tendency to partition from water into an organic phase, but is determined by organism-specific uptake, excretion and degradation rates. Furthermore, the sediment-dwellers will only take up the bioavailable

fractions of the contaminants, i.e., organotins that are freely dissolved or bound to particles that may be ingested.

The bioavailability of neutral hydrophobic contaminants in sediments is primarily governed by interactions with solid or dissolved organic matter [16]. The importance of the dissolved organic matter (DOM) in the interstitial water for distribution, mobility and bioavailability of neutral hydrophobic compounds has been demonstrated in several recent studies [17-19]. In the case of TBT and TPT, the interactions with DOM are strongly pH-dependent, and exhibit a maximum near the pK_a (Figure 4–1 [20]). Hence, under field conditions, it is very difficult to predict the bioavailable fraction. This fact is illustrated by the high variability of field-determined sediment-water distribution ratios of TBT, which range over several orders of magnitude, from < 1000 L kg^{-1} [21] to > 100'000 L kg^{-1} [22].

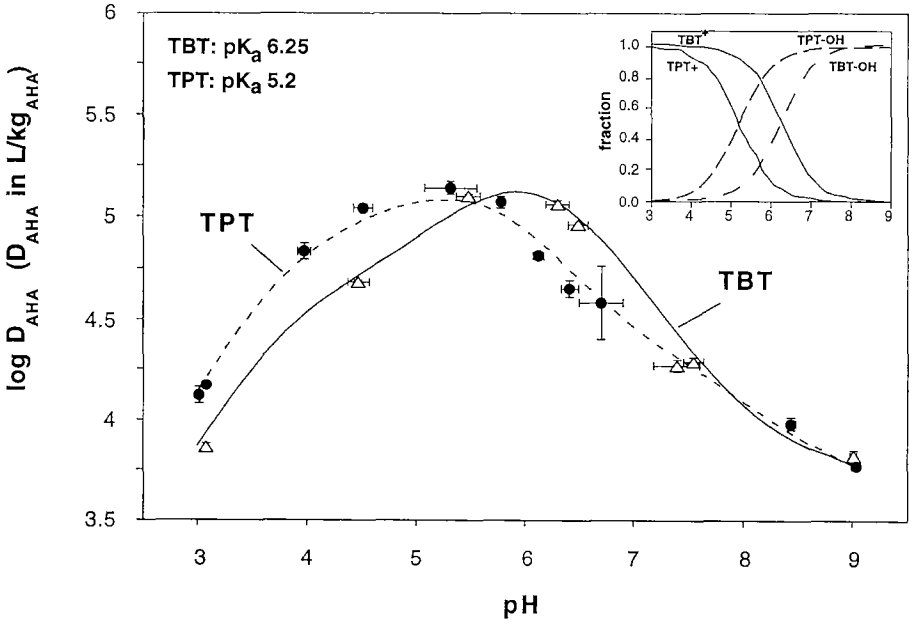

Figure 4–1. AHA-water distribution ratios (D_{AHA}) of TBT and TPT determined in dialysis experiments at 10 mM $NaClO_4$. Adapted from ref. [20]. The insert shows the pH-dependence of the triorganotin speciation at 10 mM $NaClO_4$, based on the data taken from ref. [15]. The pK_a values are 6.25 and 5.2 for TBT and TPT, respectively [15].

The objectives of the study described in this paper were (1) to obtain quantitative toxicokinetic data for TBT and TPT bioconcentration in a

representative freshwater sediment organism, (2) to investigate the effect of DOM on the bioavailability of TBT and TPT, using Aldrich humic acid (AHA) as model DOM, and (3) to evaluate the suitability of sediment organisms as biosensors for the TOT speciation in aquatic environments. To this end, we selected larvae of the non-biting midge *Chironomus riparius* as test organisms. They live in the surface layers of soft sediments and are members of the most widely distributed insect family in freshwater environments [23]. Uptake and elimination experiments with TBT and TPT were performed at pH 5 and pH 8, in order to cover maximum differences in the association of the compounds with AHA (Figure 4–1). A model with two conceptual pools (Figure 4–2) was applied to describe the time-course of TOT and metabolite concentrations in the larvae, and to derive apparent AHA-water distribution ratios (D_{AHA}), which were compared to D_{AHA} values determined in dialysis experiments [20]. The results of this study contribute to a better understanding of the factors that control the bioavailability of TOTs present in sediment pore waters and they are important for assessing the risks associated with TBT- and TPT contaminated sediments.

4.2 Materials and Methods

4.2.1 Chemicals

Tributyltin chloride TBTCl (> 97 %), dibutyltin dichloride $DBTCl_2$ (~ 97 %), and triphenyltin chloride TPTCl (> 97) were obtained from Fluka Chemie AG (Buchs, Switzerland). Diphenyltin dichloride $DPTCl_2$ (> 98 %) was purchased from ABS (Basel, Switzerland). Butyltin trichloride $MBTCl_3$ (~ 95 %), phenyltin trichloride $MPTCl_3$ (~98 %), and Amberlite IR-120(plus) ion exchange resin were obtained from Aldrich, Steinheim (Germany). The perdeuterated organotin compounds were synthesized in our laboratories [24]. Sodium tetraethylborate ($NaBEt_4$; > 98 %) was obtained from Strem Chemicals (Bischheim, France).

4.2.2 Humic Acid

The purified Aldrich humic acid (AHA) was identical with the one described in a previous study [20], and contained 57.8 % organic carbon. A detailed characterization is given in [20]. AHA stock solutions were freshly prepared for each experiment by dissolving 200 mg AHA under nitrogen in 250 mL quartz bidistilled water at pH 10. The solution was stirred for 30 min and then percolated over a preconditioned Amberlite IR-120(plus) column in order to exchange Na^+ ions by protons. Concentrations of 6, 12, 17 and 23 mg C/L in the exposure waters were obtained by adding equivalent amounts of the AHA stock solutions to M7 medium (reconstituted water as described by Elendt and Bias [25]).

4.2.3 Uptake and Elimination Experiments

Larvae of non-biting midge *Chironomus riparius* were reared as described by Streloke and Köpp [26] with slight modifications (see Chapter 2). Prior to exposure, the larvae were kept in fresh M7 medium over night to empty their guts. The uptake experiments were conducted in a climate chamber at 20 ± 2 °C with a 16 hours light and eight hours dark cycle. 40 – 120 fourth instar larvae (~140 - 480 mg) were exposed to TBT and TPT in 1 L glass beakers containing 400 - 900 mL of M7 medium and 0, 6, 12, 17 or 23 mg C/L Aldrich humic acid (AHA). The initial water concentrations ranged from 2.0 to 3.4 µg Sn/L (TBT) and from 2.9 to 3.3 µg Sn/L (TPT). The larvae were not fed and the containers were not aerated during the experiments. Mean oxygen saturation at the end of the experiments was 81 ± 8 %. The pH was measured in four hour intervals up to 12 hours of exposure, and then twice a day, and adjusted to the desired values of 8.0 and 5.0 with 0.5 M NaOH and 1.0 M HCl, respectively. After 4, 8, 12, 24, 48 and 72 hours, samples of 10 *Chironomus* larvae were collected, rinsed, dried on filter paper, weighed into vials, and stored at -20 °C until organotin analysis. Simultaneously, water samples (25 mL) were decanted, stored at 4 °C and processed for organotin analysis within less than 24 hours. Mass balance calculations revealed that the sum of organotin amounts found in water, larvae, particles and glass wall extracts after 72 hours ranged from 78 to 88 % of the initial quantities present in water.

For the elimination experiments, the exposed larvae were first rinsed in 60 mL M7 medium to remove fecal pellets and then immediately placed into 300 mL fresh M7 medium. Larvae and water (250 mL per sample) were sampled at 0, 4, 8 12, 24 and 72 hours and the remaining larvae were transferred into 300 mL fresh medium M7. The other handling was the same as for the uptake experiments.

4.2.4 Analytical Procedures

All reported organotin concentrations refer to Sn. Organotins in water samples were analyzed as described in [27]. Briefly, aliquots of 25 mL (uptake experiments) or 250 mL (elimination experiments) were ethylated with $NaBEt_4$ and simultaneously extracted with hexane during 12 hours. The hexane extract was analyzed by GC/MS with the specifications given in [27]. For quantification, internal standardization with perdeuterated standards was applied. The method detection limits, refering to 25 mL samples and expressed as three standard deviations of low concentrations [28], were 410, 37, 56, 340, 13, and 18 ng/L for MBT, DBT, TBT, MPT, DPT, TPT, respectively.

The chironomid samples were processed with a method developed for biota samples of small size. The method is described in detail in Chapter 3. Briefly, the larvae were thawed and flushed into conic 10 mL glass centrifuge tubes (NS 14/23) with 1 M sodium acetate and 1 M acetic acid in methanol and then homogenized with a glass stick. After addition of perdeuterated internal standards, the centrifuge tubes were sealed with glass stoppers and gently shaken for two hours on a lab shaker at 150 rpm. The suspensions were then quantitatively transferred into 100 mL volumetric flasks containing 2.9 g of NaCl and 1.6 ml of 2M NaOH to obtain a pH of 5. A final volume of 100 mL was achieved by adding Nanopure water. Finally, 1 mL of freshly prepared $NaBEt_4$ solution 2 % (w/v) and 1 mL of hexane were added, and the flasks were sealed with glass stoppers and shaken over night at 200 rpm. If the phase separation was not satisfactory, the extracts were centrifuged before GC/MS analysis. Recovery rates for individual butyl- and phenyltin compounds were determined by spiking unexposed chironomids with organotins and handling the samples as described above.

Relative recoveries, refering to the corresponding perdeuterated standards, were 107.0 ± 11 , 96.3 ± 6.8, 106.1 ± 14.5 , 98.5 ± 9.0 , 96.3 ± 2.8, and 103.9 ± 20.4 % for MBT, DBT, TBT, MPT, DPT, TPT, respectively (n = 3 for TPT, n = 6 for the other components). The method detection limits, refering to a wet weight of 40 mg per sample (11 chironomids) and three standard deviations of low concentrations [28], were 10, 20, 31, 52, 38, and 4 ng/g for MBT, DBT, TBT, MPT, DPT, TPT, respectively.

The concentrations of total organic carbon (TOC) in the AHA stock solutions and in exposure waters containing AHA were determined by high temperature combustion followed by infra red detection of CO_2, using a Shimadzu TOC-5000A total organic carbon analyzer.

4.2.5 Model for Triorganotin (TOT) Uptake, Excretion, and Metabolism

In many cases, bioconcentration data can be successfully described by a one-box model. If quantitative toxicokinetic data should be obtained from uptake as well as from elimination data sets, a more sophisticated model is required, since the elimination of many contaminants including organometallic compounds [29, 30] exhibits a biexponential time-course. Two kinetic pools is the minimum required to describe the elimination, since the contaminants are assumed to be present in different physiological compartments of the organisms [31]. Radiolabeled TBT, for example, was distributed very heterogenously in a marine crab after dietary uptake [30]. Based on these findings, we established a model with two conceptual pools for the tri-, di-, and monoorganotin compounds in *Chironomus riparius* larvae. As depicted in Figure 4–2, uptake, excretion and metabolism were assigned to a first pool ($c_{tri\text{-}1}$, $c_{di\text{-}1}$, and $c_{mo\text{-}1}$), whereas the second pool ($c_{tri\text{-}2}$, $c_{di\text{-}2}$, and $c_{mo\text{-}2}$) had a storage function. To minimize the number of adjustable parameters, it was assumed that the transfer rates between the two pools ($k_{trans\text{-}12}$ and $k_{trans\text{-}21}$) were identical for all butyl- and phenyltin compounds. The concentration of di- and monoorganotin compounds in the water were very low, and their uptake from water was neglected.

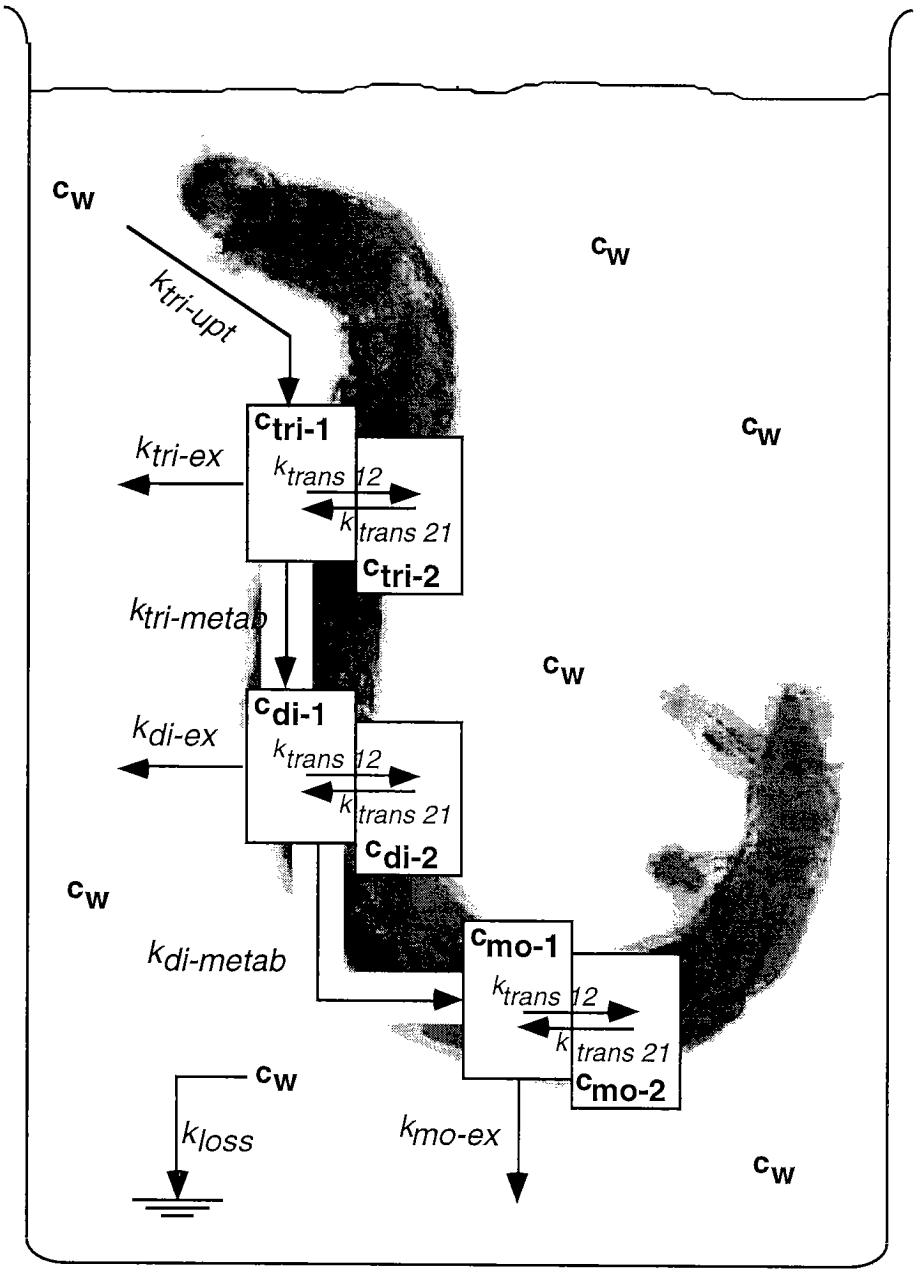

Figure 4–2. Applied model for triorganotin uptake and elimination in *Chironomus riparius* larvae. Note that the pools in the larvae are conceptual and do not necessarily correspond to physiological compartments. c_w triorganotin in water, c_{tri-1} triorganotin in pool 1, c_{tri-2} triorganotin in pool 2, c_{di-1} diorganotin in pool 1, c_{di-2} diorganotin in pool 2, c_{mo-1} monoorganotin in pool 1, c_{mo-2} monoorganotin in pool 2. **Rate constants**: k_{loss} loss by sorption, $k_{tri-upt}$ triorganotin uptake, k_{tri-ex} triorganotin excretion, $k_{tri-metab}$ triorganotin metabolism, k_{di-ex} diorganotin excretion, $k_{di-metab}$ diorganotin metabolism, k_{mo-ex} monoorganotin excretion, $k_{trans-12}$ transfer from pool 1 to pool 2, $k_{trans-21}$ transfer from pool 2 to pool 1.

According to Figure 4–2, the organotin concentrations in the system, i.e., in larvae and water, were described by a set of seven differential equations:

$$\frac{dc_{tri-1}}{dt} = k_{tri-upt}c_w + k_{trans-21}c_{tri-2} - (k_{tri-ex} + k_{tri-metab} + k_{trans-12})c_{tri-1} \quad (1)$$

$$\frac{dc_{tri-2}}{dt} = k_{trans-12}c_{tri-1} - k_{trans-21}c_{tri-2} \quad (2)$$

$$\frac{dc_{di-1}}{dt} = k_{tri-metab}c_{tri-1} + k_{trans-21}c_{di-2} - (k_{di-ex} + k_{di-metab} + k_{trans-12})c_{di-1} \quad (3)$$

$$\frac{dc_{di-2}}{dt} = k_{trans-12}c_{di-1} - k_{trans-21}c_{di-2} \quad (4)$$

$$\frac{dc_{mo-1}}{dt} = k_{di-metab}c_{di-1} + k_{trans-21}c_{mo-2} - (k_{mo-ex} + k_{trans-12})c_{mo-1} \quad (5)$$

$$\frac{dc_{mo-2}}{dt} = k_{trans-12}c_{mo-1} - k_{trans-21}c_{mo-2} \quad (6)$$

$$\frac{dc_w}{dt} = k_{tri-ex}c_{tri-1} - (k_{tri-upt} + k_{loss})c_w \quad (7)$$

where $c_{tri\text{-}1}$ and $c_{tri\text{-}2}$ [ng/mL] denote the TOT concentrations per total system volume in the first and second pool, respectively, c_w is the TOT concentration in water [ng/mL], and $k_{tri\text{-}upt}$, $k_{trans\text{-}21}$, $k_{tri\text{-}ex}$, $k_{tri\text{-}metab}$, and $k_{trans\text{-}12}$ [h^{-1}] are the first-order rate constants for TOT uptake, transfer from pool 2 to pool 1, direct excretion, metabolism, and transfer from pool 1 to pool 2, respectively. Correspondingly, $c_{di\text{-}1}$, $c_{di\text{-}2}$, $c_{mo\text{-}1}$ and $c_{mo\text{-}2}$ [ng/mL] denote the di- and monoorganotin concentrations in the two pools. The rate constants $k_{di\text{-}ex}$, $k_{mo\text{-}ex}$, $k_{di\text{-}metab}$, and k_{loss} reflect direct di- and monorganotin excretion, diorganotin metabolism, and TOT loss by sorption to the glass wall, respectively. For comparison of the calculated concentrations in the two pools with the measured total concentrations in the larvae, the ratio total volume : volume of larvae had to be considered:

$$c_{tri-tot} = (c_{tri-1} + c_{tri-2})\frac{V_{tot}}{V_{larvae}} \quad (8)$$

$$c_{di-tot} = (c_{di-1} + c_{di-2})\frac{V_{tot}}{V_{larvae}} \quad (9)$$

$$c_{mo-tot} = (c_{mo-1} + c_{mo-2})\frac{V_{tot}}{V_{larvae}} \quad (10)$$

where $c_{tri\text{-}tot}$, $c_{di\text{-}tot}$, $c_{mo\text{-}tot}$ denote the total organotin concentrations per mass of larvae [ng/g larvae], V_{tot} the total volume of the system [mL] and V_{larvae} the volume of the larvae [mL]. It was assumed that V_{larvae} corresponds to the mass of the larvae (1 g larvae ~1 mL). Note that $c_{tri\text{-}tot}$, $c_{di\text{-}tot}$, $c_{mo\text{-}tot}$ and c_w

are the four variables that represent the measured quantities during the experiments. All numerical calculations were performed with the program AQUASIM [32]. The first-order rate constants $k_{tri\text{-}upt}$, $k_{tri\text{-}ex}$, $k_{tri\text{-}metab}$, $k_{di\text{-}ex}$, $k_{di\text{-}metab}$, $k_{mo\text{-}ex}$, $k_{trans\text{-}12}$, $k_{trans\text{-}21}$, and k_{loss} were determined by simultaneous parameter estimation using the measured TOT concentrations in water and in larvae during uptake and elimination experiments at the given pH (9 parameters estimated with 64 data points).

To evaluate the results of the experiments with Aldrich humic acid (AHA), we hypothesized that TOTs were not bioavailable, when associated with AHA. The modeling of the data was based on the first-order rate constants of the controls without AHA, but f_w was introduced as adjustable parameter:

$$c_{w-free} = f_w c_w \tag{11}$$

where $c_{w\text{-}free}$ [ng/mL] is the freely dissolved TOT concentration, f_w [-] is the fraction of TOTs that are not associated with AHA, and c_w [ng/mL] the total TOT concentration in the water. The fraction f_w is a function of the AHA-water distribution ratio (D_{AHA} ; [L/kg]) at the given pH (see Figure 4–1), and of the total organic carbon concentration (TOC; [kg/L]), originating from AHA:

$$f_w = \frac{1}{1 + 1.73 D_{AHA}[TOC]} \tag{12}$$

Note that the factor 1.73 in eq 12 normalizes the distribution ratio to the organic carbon content of AHA (57.8 %). Rearrangement of eq 12 yields

$$D_{AHA} = \frac{1 - f_w}{1.73 f_w [TOC]} \tag{13}$$

The f_w values were obtained by parameter estimation according to eqs 1, 2, 7, 8, 11, and 12, using the data of the experiments with AHA. The apparent AHA-water distribution ratios (D_{AHA}, calculated with eq 13) were compared to D_{AHA} values directly determined by sorption experiments using a dialysis method [20].

4.3 Results and Discussion

4.3.1 Uptake and Elimination of TBT and TPT in the Absence of AHA

Figures 4–3 and 4–4 show the experimental data of the uptake and elimination experiments conducted with TBT and TPT in the absence of AHA. The solid lines indicate the results of the simultaneous fits of uptake and elimination data using the model discussed above. Considering that the data sets used comprise experimental data determined at very different times with different batches of organisms, the obtained fits are very satisfactory. Furthermore, the data obtained are consistent with observations made in an earlier preliminary study [33]. Hence, the parameters derived from the model fits (Table 4–1) can be looked at with some confidence.

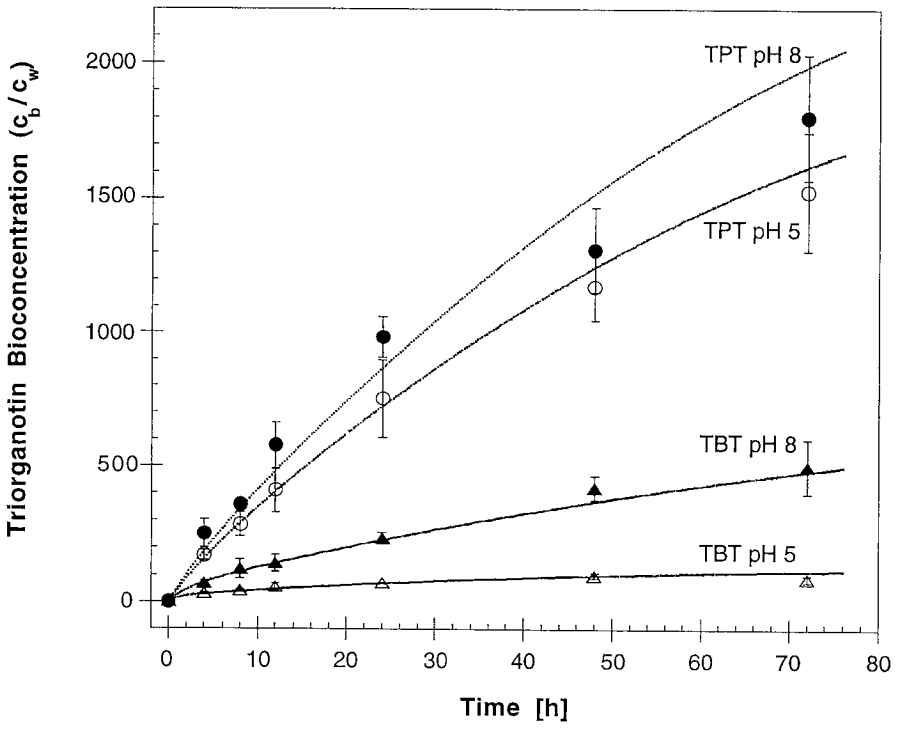

Figure 4–3. Bioconcentration of TBT and TPT in *C. riparius* larvae at pH 5 and pH 8. The larvae were exposed to initial concentrations of 2.6 ± 0.5 µg Sn/L TBT and 3.3 ± 0.2 µg Sn/L TPT. Data are given as mean ± SD (n = 3) and were fitted with the model illustrated in Figure 4–2 and described by eqs 1 to 10.

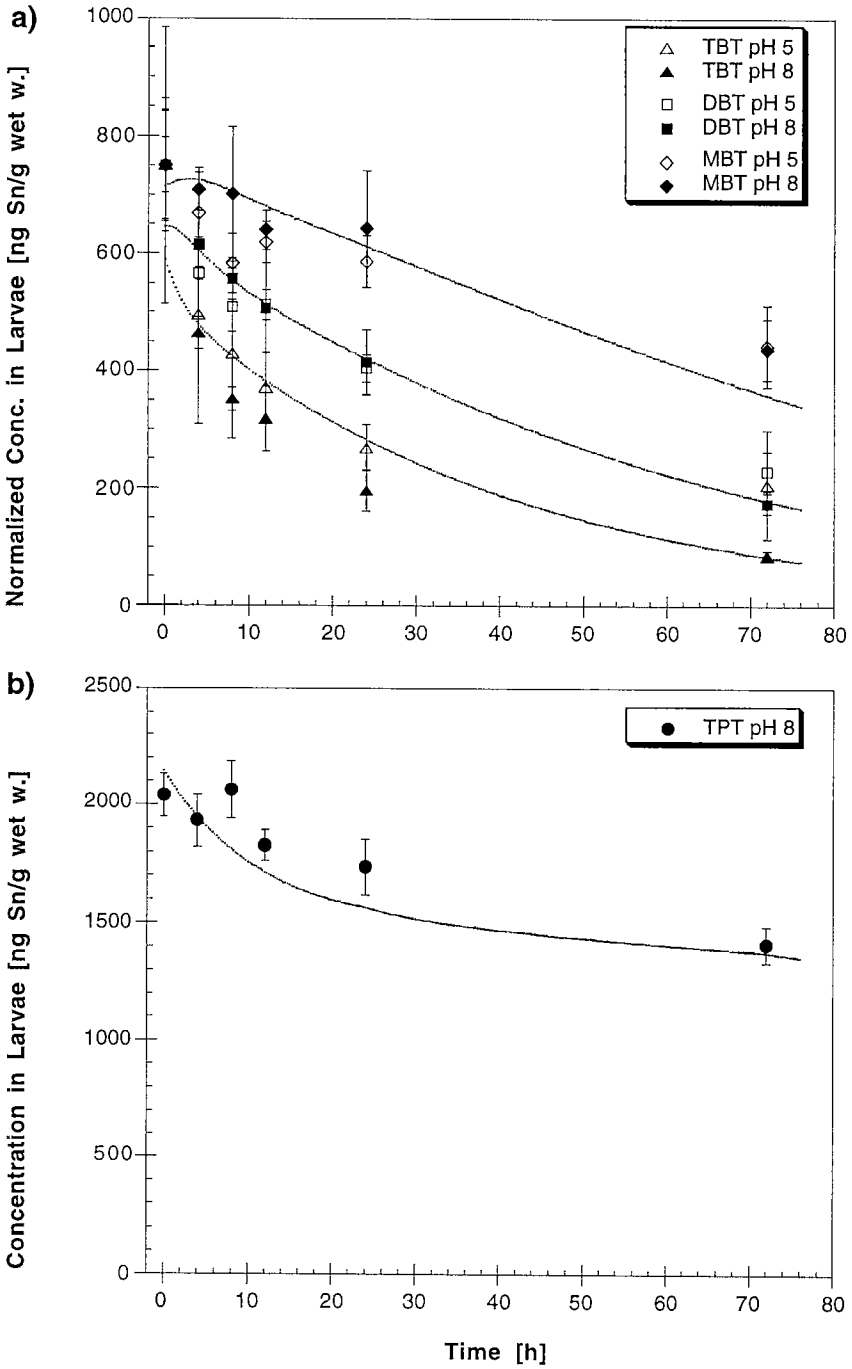

Figure 4–4. Elimination of butyltins (a) and triphenyltin (b) by *C. riparius* larvae after exposure to 3.4 ± 0.2 µg Sn/L TBT and 2.9 ± 0.1 µg Sn/L TPT for 48 hours. Clean water was provided after 4, 8, 12 and 24 hours. The individual butyltin data for pH 5 and pH 8 in (a) were normalized to initial concentrations of 750 ng Sn/g wet weight each. Data are given as mean ± SD (n = 4) and were fitted with the model described by eqs 1 to 10. Only the fits for the pH 8 data were plotted in (a). The detected traces of diphenyltin and monophenyltin were not plotted in (b).

Table 4–1. Speciation, Octanol-Water Distribution Ratios, Toxicokinetics, and Bioconcentration Factors of TBT and TPT in Experiments with *Chironomus riparius* Larvae

compound/ exposure	speciation [a]		oct.-water distr. ratio	uptake	excretion	metabolism	steady-state bioconc.f.	$BCF_{ss,max}$ [b,d] without metabolism	BCF_{ss} at 23 mg C/L AHA [b]
	$\alpha_{TOT}+$	α_{TOTOH}	D_{OW} [a]	$k_{tri\text{-}upt}$ $[h^{-1}]$ [b]	$k_{tri\text{-}ex}$ $[h^{-1}]$ [b]	$k_{tri\text{-}metab}$ $[h^{-1}]$ [b]	BCF_{ss} [b, c]		
TBT / pH 5	0.95	0.05	630	0.008 ± 0.001	0.516 ± 0.158	0.331 ± 0.079	140	210	50
TBT / pH 8	0.02	0.98	12600	0.012 ± 0.001	0.018 ± 0.020	0.324 ± 0.019	900	25000	100
TPT / pH 5	0.61	0.39	1000	0.018 ± 0.001	0.103 ± 0.025 [e]	0.003 ± 0.001 [e]	2200	2220	620
TPT / pH 8	< 0.01	> 0.99	3200	0.022 ± 0.002	0.103 ± 0.025	0.003 ± 0.001	2660	2680	1500

[a] $\alpha_{TOT}+$ fraction of triorganotin cation, α_{TOTOH} fraction of triorganotin hydroxide, data from ref [15], see insert of Figure 4–1. [b] From numerical simulations (eqs 1 to 10). [c] Lipid-normalized BCF_{ss} are 150'000 for TBT at pH 8 and 440'000 for TPT at pH 8 (calculated with a lipid fraction of 0.006 $g_{fat}/g_{Chironomus}$,from ref. [33]). [d] $k_{tri\text{-}metab}$ set equal to zero. [e] From experiments performed at pH 8.

Inspection of Figure 4–3 and Table 4–1 reveals some interesting features. First, at pH 5 as well as at pH 8, bioconcentration of TPT was significantly higher as compared to TBT. This is remarkable, since both TBT^+ and TBTOH are more hydrophobic than TPT^+ and TPTOH, as is reflected in their octanol-water distribution behavior (D_{OW} in Table 4–1, from ref [15]). The major reason for these findings is the significant metabolism in *Chironomus riparius*, whereas for TPT, the only important elimination mechanism was excretion. In fact, when setting $k_{tri\text{-}metab}$ equal to zero, at pH 8, where TBTOH and TPTOH are the dominant species (see α_{TOTOH} values in Table 4–1), the model calculations yield steady-state bioconcentration factors that reflect very well the relative hydrophobicities of the two species (compare D_{OW} values in Table 4–1; note that at pH 8, $D_{OW} \sim K_{OW}$ of the TOTOH species).

At pH 5, the situation is somewhat more complicated. As indicated in Table 4–1, at this pH, TBT is present primarily as positively charged TBT^+ (95 %), whereas in the case of TPT, both species are present at about equivalent fractions (61 % and 39 %, respectively). Inspection of the D_{OW} values of the two compounds (Table 4–1) reveals that only the partitioning of the neutral species is important in the octanol-water system. When assuming that this is also true for the bioconcentration in *Chironomus riparius*, steady-state bioconcentration factors (BCF_{ss}) at pH 5 of (0.05)(900)=45 for TBT and (0.39)(2660)=1040 for TPT would be expected. The experimental values are, however, 140 and 2200, respectively (Table 4–1). This suggests that not only the neutral TOTOH but also the charged TOT^+ species are taken up by *Chironomus riparius* larvae. When assuming that the BCF_{ss} at a given pH can be expressed by

$$BCF_{ss} = \alpha_{TOT^+} BCF_{ss,TOT^+} + \alpha_{TOTOH} BCF_{ss,TOTOH} \qquad (14)$$

and by using $BCF_{ss,TOTOH} = BCF_{ss}\ (pH\ 8)$, one can calculate BCF_{ss,TOT^+} values for TBT and TPT from the experimental data at pH 5 and 8 by

$$BCF_{ss,TOT^+} = \frac{BCF_{ss}(pH5) - \alpha_{TOTOH}(pH5) BCF_{ss}(pH8)}{\alpha_{TOT^+}(pH5)} \qquad (15)$$

The values obtained are 100 for TBT^+ and 1900 for TPT^+, yielding BCF_{ss,TOT^+} / $BCF_{ss,TOTOH}$ ratios of 0.11 and 0.72, respectively, for the two compounds. Some important conclusions can be drawn from these findings.

First, in the case of TPT, the charged species is taken up to almost the same extent as the neutral species, which is completely different from what one would predict from the octanol-water distribution behavior of the component (see above). Second, the uptake of TBT^+ is almost one order of magnitude smaller as compared to TBTOH, but is not negligible. Third, and most interestingly, the $BCF_{ss,TOT+}$ / $BCF_{ss,TOTOH}$ ratios of 0.11 and 0.72 indicate that relative to the bioconcentration of the neutral species (which reflects primarily hydrophobic partitioning), TPT^+ is taken up about 7 times more efficiently than TBT^+. This difference between TPT^+ and TBT^+ corresponds very closely to the difference in the tendency postulated for the two species for formation of innersphere complexes with carboxyl and phenol groups present in natural organic matter [20]. Note that this higher affinity of TPT^+ to oxygen ligands is also reflected in the pK_a's of the two components (i.e., 5.2 versus 6.25). Hence, one could speculate that the major mechanism of the uptake of the TOT^+ species *by Chironomus riparius* larvae is binding to O-ligands. The much weaker tendency of TBT^+ to bind to such ligands would also explain the much higher excretion rate found for TBT at pH 5 as compared to pH 8.

4.3.2 TBT Metabolism

As already pointed out above, no significant metabolism of TPT was observed. In contrast, as shown by the rate constants given in Table 4–2, TBT was rapidly debutylated to DBT, which was then converted to MBT. The debutylation rate constants were similar at pH 5 and pH 8. Note that for the metabolism of MBT, no rate constant can be derived from the data because inorganic tin was not determined. The TBT half-lifes of 16 and 22 hours in *Chironomus* larvae were very short, whereas in seven fish and three mussel species, the half-lifes ranged from 2.0 to 69 days [7, 34-36, 37, 38, 39]. We hypothesize that the efficiency of the metabolism and the small size of the larvae, resulting in a low diffusion path length [40], are the major reasons for the differences between *Chironomus* and other organisms. We did not study the distribution of the metabolites in the larvae tissues, but from studies with other arthropods such as crabs and shrimps [41], it can be assumed that metabolic activity is highest in the hepatopancreas.

Table 4–2. Characteristics of Butyltin Elimination in *C. riparius*

compound/ conditions	excretion rate constant [h^{-1}] [a]	debutylation rate constant [h^{-1}] [a]	ratio debutylation : excretion [b]	half-life in *C. riparius* [h] [a]	time for 95% elimination [d] [a]
TBT / pH 5	0.516 ± 0.0158 [c]	0.331 ± 0.079 [f]	0.6	16	3.9
TBT / pH 8	0.018 ± 0.020 [c]	0.324 ± 0.019 [f]	18	22	4.7
DBT / pH 5	0.057 ± 0.024 [d]	0.139 ± 0.043 [g]	2.4	34	6.4
DBT / pH 8	0.049 ± 0.005 [d]	0.187 ± 0.016 [g]	3.8	39	6.7
MBT / pH 5	0.076 ± 0.043 [e]		-	102	13
MBT / pH 8	0.174 ± 0.018 [e]		-	68	9.1

[a] From numerical simulations (eqs 1 to 10). The transfer rates between the two pools in the model were $k_{trans\text{-}12}$ 0.117 ± 0.010 and $k_{trans\text{-}21}$ 0.035 ± 0.003. [b] Relative importance of debutylation and excretion processes. [c] $k_{tri\text{-}ex}$, for convenience listed in both Table 4–1 and Table 4–2. [d] $k_{di\text{-}ex}$. [e] $k_{mo\text{-}ex}$; note that this rate constant includes excretion and possible debutylation to inorganic tin. [f] $k_{tri\text{-}metab}$, for convenience listed in both Table 4–1 and Table 4–2. [g] $k_{di\text{-}metab}$.

4.3.3 Effect of Aldrich Humic Acid (AHA) on TBT and TPT Bioconcentration

As shown by Figure 4–1, sorption of TBT and TPT to AHA exhibits a maximum at the pH corresponding to the pK_a of the given compound, since TBT^+ and TPT^+ form specific complexes with the acidic groups (carboxyl groups, phenolic groups). Note that the D_{AHA} values shown in Figure 4–1 have been determined by dialysis experiments in a 10 mM $NaClO_4$ electrolyte, whereas the M7 medium for the bioconcentration experiments contained significant concentrations of Ca^{2+} (2mM) and Mg^{2+} (0.5mM). These bivalent cations may influence the sorption of the TOTs in two ways. On the one hand, they may compete with the cationic TOT species for the carboxylate and phenolate groups of the dissolved humic acid [42], thus decreasing overall sorption. On the other hand, particularly at higher pH value (i.e., pH $\geq$ 8), the presence of bivalent cations may alter the conformation of dissolved humic acids such that additional hydrophobic domains are created [43, 44]. This effect would favor the hydrophobic partitioning component of the overall D_{AHA} value of a given TOT. Thus, for the following comparison of D_{AHA} values determined by dialysis experiments with apparent D_{AHA} values derived from the bioconcentration experiments with *Chironomus riparius* (eq 13), these differences in experimental conditions should be kept in mind.

Figure 4–5 shows the experimental data of the uptake experiments in the presence of 23 mg C/L AHA. The solid lines indicate the results of the model fits using the uptake, excretion and metabolism rate constants derived from the experiments without AHA (Table 4–1), and the fraction in dissolved form, f_w (eq 11 and 12) as the sole fitting parameter. The resulting steady-state bioconcentration factors are given in Table 4–1. As is evident from Figure 4–5, the experimental data could be very well described by the model, considering that only one fitting parameter was used. Analogous results were obtained for the experiments with AHA concentrations of 6, 12, and 17 mg C/L (data not shown).

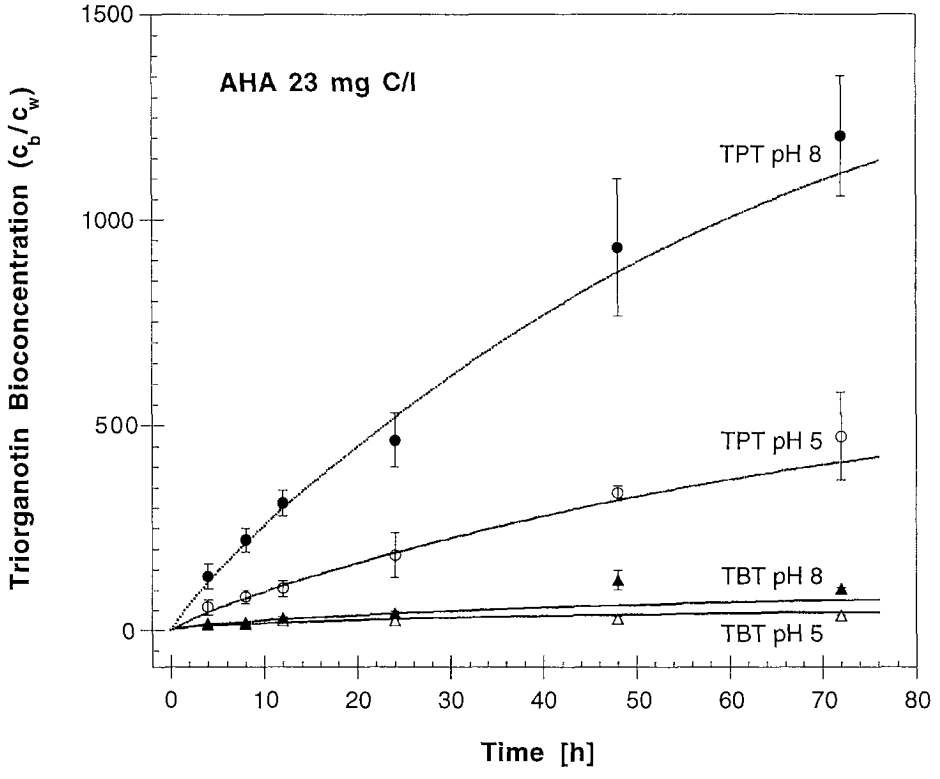

Figure 4–5. Bioconcentration of TBT and TPT in *C. riparius* larvae at pH 5 and pH 8 and in the presence of 23 mg C/L AHA. The larvae were exposed to initial concentrations of 2.4 ± 0.4 µg Sn/L TBT and 3.0 ± 0.2 µg Sn/L TPT. Data are given as mean ± SD (n = 3), but some error bars lie within the markers. The bioconcentration curves were calculated with the rate constants from experiments without AHA (Table 4–1), using the fraction of freely dissolved triorganotin (f_w) as adjustable parameter (eqs 1, 2, 7, 8, 11, and 12).

Comparison of Figures 4–3 and 4–5, and of the corresponding BCF_{ss} values given in Table 4–1 shows that the presence of 23 mg C/L AHA led to a significant decrease in the bioavailability and thus in the degree of bioconcentration of TBT and TPT at both pH 5 and pH 8. In Table 4–3, the average apparent D_{AHA} values derived from the bioconcentration experiments with 6, 12, 17, and 23 mg C/L AHA are compared to D_{AHA} values directly determined using the classical dialysis method [20]. As can be seen, except for TBT at pH 8, a very good agreement between the two sets of D_{AHA} values was found. The somewhat lower D_{AHA} values derived from the bioconcentration experiments at pH 5 could be due to the presence of competing Ca^{2+} and Mg^{2+} ions (see above). The significantly and reproducibly higher D_{AHA} value found for TBT at pH 8 is somewhat more

difficult to rationalize. One plausible explanation is the formation of hydrophobic domains in the AHA due to the presence of Ca^{2+} and Mg^{2+} (as compared to Na^{+} in the dialysis experiments). The hydrophobic contribution to the overall sorption to AHA is presumably much more important for TBT as compared to TPT [20], which could explain the observed discrepancies. Nevertheless, these findings are consistent with the hypothesis that organic pollutants associated with dissolved organic matter are not bioavailable [45].

Table 4–3. Comparison of AHA-Water Distribution Ratios (D_{AHA}) of TBT and TPT Determined by Dialysis with those derived from the Bioconcentration Experiments with *Chironomus riparius*

compound/ conditions	log D_{AHA} dialysis experiment [a] data from ref. [20]	log D_{AHA} bioconc. experiment [b] eq 13
TBT / pH 5	4.90 ± 0.04	4.68 ± 0.09
TBT / pH 8	4.08 ± 0.09	4.87 ± 0.06
TPT / pH 5	5.06 ± 0.07	4.64 ± 0.09
TPT / pH 8	4.08 ± 0.09	4.13 ± 0.04

[a] D_{AHA} values and standard deviations obtained from data depicted in Figure 4–1 [20]). [b] Determined in bioconcentration experiments with 6, 12, 17, and 23 mg C/L, according to eqs 1, 2, 7, 8 and 11-13.

4.4 Environmental Significance

The results of this study demonstrate that larvae of *Chironomus riparius* are well suited organisms to study the bioavailability and bioconcentration of organotin compounds from aqueous solution. We have also shown that by using a rather simple two-pool model, all relevant parameters including uptake, excretion and degradation rates, as well as the bioavailability as a function of pH and concentration of dissolved organic matter can be derived with very good reproducibility.

With respect to the compounds studied, it was found that despite its greater hydrophobicity, due to an efficient metabolism, bioconcentration of TBT by *Chironomus riparius* was up to more than an order of magnitude lower as compared to TPT. TBT metabolism in chironomids has already been suggested in a comprehensive field study [46]. Since chironomids are an important food source for fish and birds [23] and TBT uptake via food may be an important accumulation route [30], *Chironomus* species with fast TBT metabolism relieve their predators from high TBT uptake. In addition to chironomids, the butyltin patterns found in gammarids [46], fish [46], birds [46], river otters [47], and marine mammals [48-51] illustrate the presence of TBT metabolism from low to top trophic levels. In contrast, reports of phenyltin concentrations in field-collected organisms are scarce, but slow metabolism of TPT was also suggested for zebra mussels [52], horseshoe crabs [53], and several fish species [46]. Thus, considering the absence of a significant metabolism and the relatively high bioconcentration of TPT in organisms such as chironomids, the hazard potential of TPT in aquatic systems should be more carefully reevaluated. Finally, for both TBT and TPT, uptake by ingestion of particle bound compound should be included into the considerations. Such work is presently in progress in our laboratory.

4.5 Acknowledgments

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5

Tributyltin Uptake by *Chironomus riparius* in Reference Sediment PACS-2: Preliminary Experiments to Evaluate the Relative Importance of the Pore Water and the Sediment Ingestion Pathway

Chironomus riparius larvae were exposed to tributyltin (TBT) in water and in reference sediment PACS-2. The PACS-2 exposed larvae showed a higher variability in the TBT concentrations than the water exposed larvae. TBT uptake via ingestion of sediment particles contributed significantly to the total TBT body burden, and both uptake from the sediment pore water and uptake via sediment ingestion were found to be of similar magnitude. The results of these preliminary experiments indicate that the feeding behavior of organisms cannot be neglected, when the hazard potential of sediment associated TBT is discussed.

5.1 Introduction

The toxicity of organotin compounds towards non-target organisms in aquatic ecosystems, in particular of tributyltin (TBT), has already been recognized two decades ago [1]. To date, legal restrictions on the use of TBT-based antifouling paints have been implemented worldwide [2, 3] and the international maritime organization plans a total TBT ban for antifouling paints by 2003 [4]. In general, the measured TBT concentrations in the water column responded rapidly to the reduced TBT inputs [5], and very high TBT concentrations are typically limited to sediments, where the residence times are considerably longer [6-8]. Whether or not these contaminated sediments pose a risk to benthic organisms is strongly dependent on the bioavailability of the TBT for the sediment-dwellers.

As is illustrated in Figure 5-1, three potential uptake paths have to be considered, i.e., uptake from the overlying water, uptake from the pore water, and ingestion of sediment particles. For burrowing midge larvae of the chironomidae family, which are considered as key members im many benthic communities [9], uptake from the pore water, and ingestion of sediment particles are the relevant exposure routes. It is evident that a strong association of the TBT with the solid sediment lowers the uptake from water, but simultaneously enhances the relevance of the particle ingestion pathway.

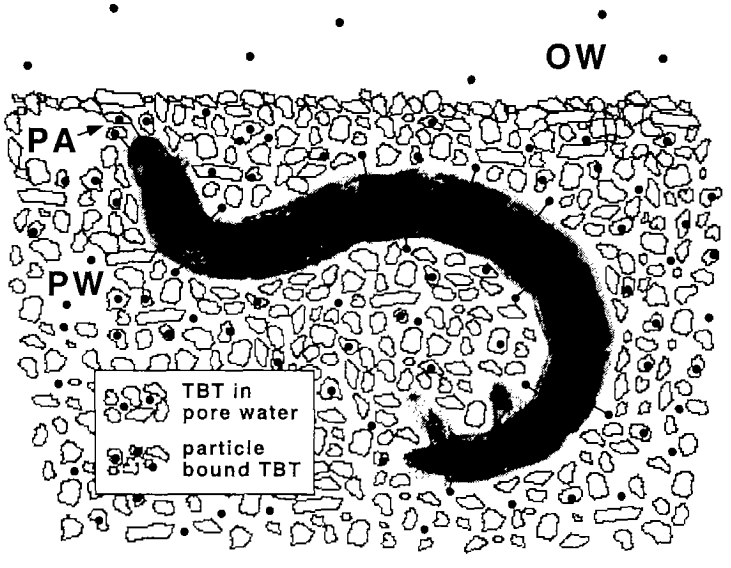

Figure 5–1. Schematic representation of a *Chironomus* larvae in sediment and routes of exposure to TBT. OW Overlying water. PW Pore water. PA Sediment particles.

Hitherto, little attention has been paid to the possibility of TBT uptake via sediment particles. Considering that representative freshwater sediments are characterized by sediment-water distribution ratios of higher than 1000 and water contents of ~ 70 % [10], one can assume that more than 99 % of the total TBT is bound to the particles and less than 1 % dissolved in the pore water. Since chironomids feed on all components of the surface sediment, including detritus, algae, and mineral components [11], a significant TBT uptake via sediment particles has to be expected. It has been shown in Chapter 4 that dissolved organic matter lowers the bioavailability of TBT in aqueous systems. The concentrations of dissolved organic matter (DOC) in pore waters of freshwater harbor sediments are typically between 5 and 10 mg C/L [10]. Hence, not the total TBT in the pore water will be bioavailable. It should be kept in mind that this factor may lower the importance of the pore water uptake route, as compared to sediment ingestion.

The objective of the preliminary experiments described in this chapter was to evaluate the TBT uptake by *Chironomus riparius* larvae via sediment particles and to compare the importance of the pore water and the sediment ingestion pathways. Therefore, the larvae were exposed to a reference sediment with a certified TBT concentration (PACS-2) and with a known sediment-water distribution ratio for TBT [10]. The TBT bioconcentration with respect to the total concentration in the pore water was compared to the bioconcentration in a control experiment with a TBT solution that was free of sediment particles. The particle-bound TBT uptake was estimated on the base of the model described in Chapter 4, by introducing a sediment ingestion factor λ as adjustable parameter.

5.2 Experimental Section

5.2.1 Materials

Reference sediment PACS-2 was obtained from Promochem GmbH, Wesel, Germany. The material was originally collected in the harbor of Esquimalt, British Columbia, freeze dried, and sieved (125 µm mesh) before blending and bottling. The total carbon content was determined in our

laboratories with a CARLO ERBA Elemental Analyzer EA 1108 by combustion to CO_2, separation by gas chromatography and detection by a thermal conductivity detector (TCD). Inorganic carbon was determined by converting the carbonate of the sediment into CO_2 with HCl and subsequent titration of the CO_2 with a Coulometer (Coulometrics Inc.). The organic carbon content was obtained as the difference of the total carbon and the inorganic carbon. A characterization of the PACS-2 reference sediment is given in Table 5–1. All other materials used for this work are described in detail in Chapter 4.

Table 5–1. Carbon and TBT Contents of PACS-2, Applied Sediment-Water Distribution Ratio (K_d), and Calculated Pore Water Concentration (c_{pw}) in the Experimental System

Characteristic	PACS-2
Total carbon [%]	3.25 ± 0.03
Inorganic carbon [%]	0.10 ± 0.01
Organic carbon [%]	3.15 ± 0.05
TBT (c_s ; [ng/g]) [a]	980 ± 130
K_d [L/kg]) or [mL/g]) [b]	4160 ± 90
C_{pw} [ng/mL]) [c]	0.24 ± 0.04

[a] Certified Value. [b] From resuspension experiments [10]. [c] Calculated from c_s and K_d by eq 7.

5.2.2 Exposure and Sampling of *Chironomus* larvae

The culturing of the *Chironomus riparius* larvae is described in Chapter 2. Before starting the experiment, the larvae were kept over night in fresh M7 medium (reconstituted water as described in ref. [12]) to clean their guts. For exposure to PACS-2, 20 g of this reference material were weighed into 1 L glass beakers. 800 mL of M7 medium were added, and the sediment

surfaces were intensively aerated through pasteur pipets at ~ 5 bubbles per second. The systems were allowed to equilibrate for two hours, before 110 *Chironomus* larvae (4th instar) were transferred on the sediment surface in each beaker. The experiment was conducted in triplicate. For sampling the larvae, the overlying water was decanted into a glass beaker and after picking the larvae on the surface of the solid sediment with a pincet, the water was replaced again. Samples were taken after 4, 8, 12, 24 hours (20 larvae per sample) and after 48 and 72 hours (10 larvae per sample). The larvae were rinsed with Nanopure water, dried on filter paper, weighed, and stored in glass vials at – 20 °C until organotin analysis. The average pH of six measurements (twice a day) was 7.7 ± 0.1. For the control experiment without sediment, a triplicate of 45 *Chironomus* larvae were exposed in glass beakers with 800 mL M7 medium spiked with TBTCl (initial concentration 3.0 ± 0.3 ng Sn/mL). The control beakers were not aerated, since in previous experiments, oxygen saturation had been ≥ 80 % without aeration (see Chapter 4). The pH was measured twice a day and ajusted to 7.7 with NaOH 0.1 M, if necessary. The average pH of six measurements was 7.6 ± 0.2 . After 8, 24, 48, and 72 hours, samples of 10 larvae were collected and treated as described above. Simultaneously, 25 mL aliquots of water were removed and stored in the volumetric glass flasks at 4 °C for less than 24 hours, until processing for organotin analysis.

5.2.3 Determination of Butyltin Compounds in Larvae and Water

All reported organotin concentrations refer to Sn. The larvae samples were processed by cold methanolic digestion, aqueous derivatization with $NaBEt_4$ and simultaneous liquid-liquid extraction with hexane. The hexane extracts were analyzed using gas chromatography/mass spectrometry (GC/MS). Details of the analytical procedure are given in Chapter 3. The water samples were processed by aqueous derivatization with $NaBEt_4$, liquid-liquid extraction with hexane, and analysis of the hexane extracts by GC/MS [13].

The TBT concentrations in the pore water were not measured, but calculated from the K_d value determined in a resuspension experiment (Table 5–1). Although the resuspension experiment was performed at pH 7.3

and the *Chironomus* experiment at pH 7.7, this K_d value can serve to estimate the pore water concentration with acceptable uncertainty (for the pH-dependence of interactions with organic matter, see Figure 4–1 in Chapter 4).

5.2.4 Data Evaluation

The evaluation of the experimental data was based on the two-pool model described in Chapter 4 and illustrated in Figure 4-2. The TBT uptake by the *Chironomus* larvae of the control experiment without sediment was calculated with the following four equations (see Figure 4.2):

$$\frac{dc_{tri-1}}{dt} = k_{tri-upt}c_w + k_{trans-21}c_{tri-2} - (k_{tri-ex} + k_{tri-metab} + k_{trans-12})c_{tri-1} \quad (1)$$

$$\frac{dc_{tri-2}}{dt} = k_{trans-12}c_{tri-1} - k_{trans-21}c_{tri-2} \quad (2)$$

$$\frac{dc_w}{dt} = k_{tri-ex}c_{tri-1} - (k_{tri-upt} + k_{loss})c_w \quad (3)$$

$$c_{tri-tot} = (c_{tri-1} + c_{tri-2})\frac{V_{tot}}{V_{larvae}} \quad (4)$$

where c_{tri-1} and c_{tri-2} [ng/mL] denote the TBT concentrations per total system volume in the first and second pool, respectively, c_w [ng/mL] is the TBT concentration in water and $k_{tri-upt}$, $k_{trans-21}$, k_{tri-ex}, $k_{tri-metab}$, $k_{trans-12}$, and k_{loss} [h^{-1}] are the first-order rate constants for TBT uptake, transfer from pool 2 to pool 1, direct excretion, metabolism, transfer from pool 1 to pool 2, and loss by sorption to the glass wall, respectively. In eq 4, $c_{tri-tot}$ denotes the total TBT concentration per mass of larvae [ng/g larvae], V_{tot} is the total volume of the system [mL] and V_{larvae} the volume of the larvae [mL]. It was assumed that V_{larvae} corresponds to the mass of the larvae (1 g larvae ~ 1 mL). The values for the rate constants k_{tri-ex}, $k_{tri-metab}$, $k_{trans-12}$, and $k_{trans-21}$ were taken from the experiments described in Chapter 4, whereas $k_{tri-upt}$ and k_{loss} were determined by parameter estimation.

For the *Chironomus* larvae exposed to PACS-2 sediment, it was assumed that the mechanism of TBT uptake from the sediment pore water was the same as from the water in the control experiment and that the TBT uptake from the pore water and the TBT uptake via sediment particles were additive. Hence, eq 1 was modified to

$$\frac{dc_{tri-1}}{dt} = \lambda k_{tri-upt} c_{pw} + k_{trans-21} c_{tri-2} - (k_{tri-ex} + k_{tri-metab} + k_{trans-12}) c_{tri-1} \quad (5)$$

where c_{pw} denotes the TBT concentration in the pore water [ng/mL], and the sediment ingestion factor λ [-] accounts for enhanced TBT bioaccumulation due to uptake of particles. For $\lambda = 1$, no TBT uptake by ingestion of sediment particles occurs, whereas e.g. for $\lambda = 2$, the particle uptake route is of equal importance as uptake from the pore water.

The degree of TBT uptake by particle ingestion is a function of the TBT concentration in the solid sediment and of the amount of ingested sediment particles, i.e., the grazing rate. Scenarios on the relative importance of sediment ingestion as a function of the K_d value and at different grazing rates were calculated by defining the sediment ingestion factor λ as

$$\lambda = \frac{k_{upt,app} c_{pw} + g_r c_s}{k_{upt,app} c_{pw}} \quad (6)$$

where the apparent rate constant $k_{upt,app}$ [h^{-1}] replaces the adjustable parameter $k_{tri\text{-}upt}$ in eq 1 and 3, if $k_{tri\text{-}ex}$ and $k_{tri\text{-}metab}$ are set to zero. Note that for direct comparison of the two uptake routes, metabolism and excretion have to be excluded from the model calculations, since the different uptake paths involve different physiological compartments with unknown elimination rates. c_s is the TBT concentration in the sediment [ng/g sediment] and g_r is the grazing rate of the *Chironomus* larvae [g sediment/g larvae/h]. The TBT concentration in the pore water (c_{pw}) can be calculated by

$$c_{pw} = \frac{c_s}{K_d} \quad (7)$$

where K_d is the sediment-water distribution ratio [mL/g]. The combination of eq 6 and eq 7 results in

$$\lambda = \frac{k_{upt,app} + g_r K_d}{k_{upt,app}} \tag{8}$$

Rearrangement of eq 8 yields

$$g_r = \frac{(\lambda - 1)k_{upt,app}}{K_d} \tag{9}$$

The TBT uptake curve for the PACS-2 exposed *Chironomus* larvae was calculated according to eqs 2 to 5 and with the rate constants of the control experiment, but with the pore water concentration c_{pw} calculated from the K_d value (Table 5–1). The sediment ingestion factor λ was determined by parameter estimation. All parameter estimations and numerical simulations were performed with the program AQUASIM [14].

5.3 Results and Discussion

5.3.1 TBT Bioaccumulation in *Chironomus riparius* Larvae

Figure 5–2 shows the time-course of TBT bioaccumulation in the water and PACS-2 exposed *Chironomus* larvae, expressed as the concentrations in the larvae (c_b) divided by the concentrations in water (c_w) and in pore water (c_{pw}), respectively. Already in the first samples taken after four hours of exposure to PACS-2, the larvae showed dark coloured guts and a significant ingestion of sediment particles was evident. Considering these observations, the higher TBT bioaccumulation of the PACS-2 exposed larvae as compared to the control (Figure 5–2) was not surprising. The fitting of the PACS-2 data yielded a sediment ingestion factor of 2.0 ± 0.1, which demonstrates, that TBT uptake from pore water and via sediment ingestion were of similar magnitude. Note that the variability of TBT uptake was much higher in the more complex PACS-2-water system than in the water-only system of the control experiment.

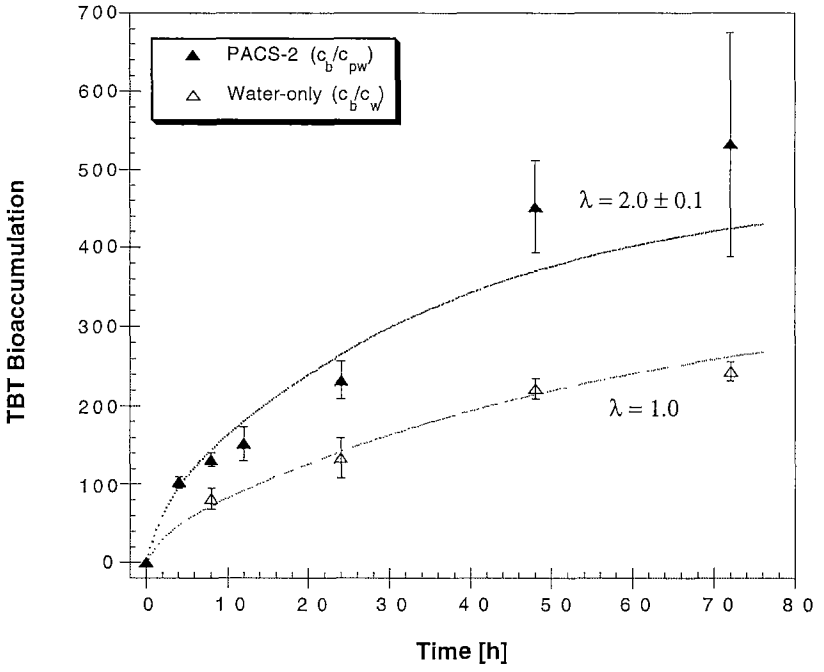

Fig. 5-2. Time-course of TBT bioaccumulation by *Chironomus riparius* larvae exposed in water (control experiment) and reference sediment PACS-2 (1 g Larvae ~ 1 mL). The data are given as mean ± SD and were fitted with the model descibed by eqs 1 to 5. The sediment ingestion factor λ for the PACS-2 exposed larvae was determined by parameter estimation (eq 5).

As can be seen in Table 5–2, the PACS-2 exposed larvae achieved lower TBT body burdens after 72 hours than the water exposed larvae. This is consistent with the lower overall mortality of the sediment-exposed larvae, although the mortality was lower than 10 % in both cases. In PACS-2, the larvae took up only 0.17 % of the total TBT in the sediment, and TBT uptake was thus not limited by the TBT amounts present in the solid sediment and in the sediment pore water.

5.3.2 Relevance of Particle Ingestion

In a recent field study with chironomids [11], an average grazing rate of 0.0051 g particles/g larvae (dry weight)/h was suggested for fourth instar *Chironomus* larvae. The dry weight of our laboratory-reared *Chironomus riparius* larvae was 12 ± 1 % of the wet weight (Chapter 2). Hence, a grazing rate of 0.0006 g particles/g larvae (wet weight)/h can be assumed. With this grazing rate and with the TBT content of 980 ng/g in PACS-2, a

TBT body burden of (0.0006)(980)(72) = 42 ng/g would be attributed to sediment ingestion after 72 hours, whereas the actually measured body burden was 133 ng/g (Table 5–2). A portion of 42 ng/g from particle ingestion and 91 ng/g from pore water would result in a λ value of 1.5, whereas the fit of the experimental data yielded a λ value of 2.0. Considering that the assumed grazing rate is an estimate derived from other *Chironomus* species and that in this work, the *Chironomus riparius* larvae had emptied their guts before exposure, the agreement between both λ values is reasonable.

Table 5-2. Characteristics of TBT Bioaccumulation for Chironomus riparius Larvae Exposed in Water and PACS-2 Sediment

Exposure	TBT body burden (72h) [ng/g] [a]	Mortality [%] [a]	Total TBT in larvae [%] [a,b]	Sediment ingestion factor λ [c]	Grazing rate g_r [g/g] [d]
Water	451 ± 23	7.4 ± 2.6	2.7 ± 0.2	1.0	0
PACS-2	133 ± 36	1.5 ± 1.4	0.17 ± 0.03	2.0 ± 0.1	0.0013

[a] Mean ± SD (n = 3). [b] Absolute amounts in sampled larvae as percentage of the total TBT amounts in water and sediment, respectively. [c] Determined by parameter estimation (eq 2–5). [d] Calculated by eq 9 with $\lambda = 2.0$, $k_{upt,app} = 5.5$ (parameter estimation), and $K_d = 4160$.

Due to the small amounts of sediment, no concentrations of dissolved organic carbon (DOC) could be determined in the pore water. As already mentioned, the DOC in the sediment pore water is expected to lower the bioavailability of TBT. Assuming equal association of TBT with the pore water DOC as with Aldrich humic acid (Chapter 4), a typical DOC of 7 mg C/l would reduce the TBT uptake from pore water by a factor 2. Consequently, the particles would contribute by a factor 2 more to the total TBT uptake than indicated by the λ value. Hence, the contribution of the particles was possibly underestimated by this approach.

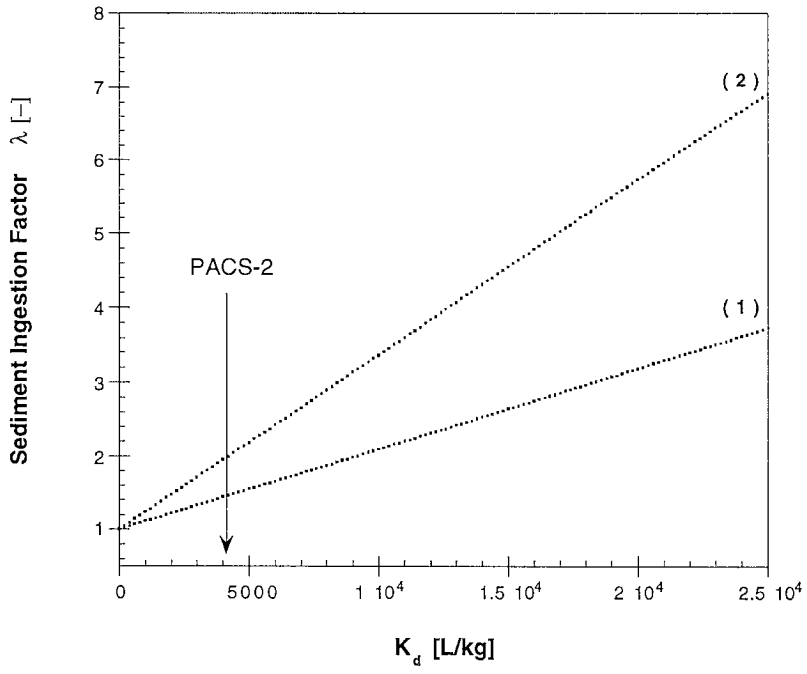

Figure 5–3. Sediment ingestion factor λ as a function of the sediment-water distribution ratio K_d with respect to different grazing rates. Both scenarios were calculated by eq 8 with $k_{upt,app}$ = 5.5 h^{-1}. Scenario (1) was calculated with a grazing rate of 0.0006 g/g/h ([11]), and scenario (2) with a grazing rate of 0.0013 g/g/h (calculated by eq 9 with λ=2.0, for PACS-2 exposed *Chironomus* larvae).

Figure 5–3 shows two scenarios on the relative importance of sediment ingestion (λ) as a function of the K_d value, calculated with a grazing rate from literature (scenario 1) and with the estimated grazing rate of Chironomus riparius exposed to PACS-2 sediment (scenario 2). It is obvious, that already for very small K_d values, i.e., ~1000, the sediment ingestion factor λ becomes ≥ 1.1 at both grazing rates and that TBT uptake via particle ingestion is not negligible as compared to the pore water uptake route. One can assume that under field conditions, the grazing rate used for scenario 1 is more realistic than the grazing rate used for scenario 2, since the *Chironomus riparius* larvae had empty guts, when the exposure to PACS-2 started. However, both scenarios suggest that for Kd's higher than 10'000, which are common in freshwater harbor sediments [10], ingested particles are expected to contribute more to the total TBT uptake as compared to the uptake from pore water. Hence, in sediments with very high Kd's, the bioavailability of TBT to *Chironomus* species will not be low

in general, because the lower uptake from pore water is linked to a higher TBT uptake via sediment particles.

5.4 Environmental Significance

This work demonstrates that TBT uptake via ingestion of sediment particles is relevant for *Chironomus riparius* larvae, and that under the given experimental conditions, TBT uptake via sediment ingestion was at least as important as uptake from pore water. A high contribution of sediment ingestion to the total uptake has recently been reported for polychlorinated dibenzo-p-dioxins [15] as well as for pyrene [16], both in sediment-dwelling oligochaete worms. In the second study, it was hypothesized that for compounds with log $K_{OW} < 5$, the major route for the accumulation is pore water [16]. If only the hydrophobicity of TBT would be considered (log K_{OW} 4.1, of the neutral TBTOH species [17]), a low contribution of ingested particles to the total TBT uptake would be expected. In contrast, the PACS-2 exposed *Chironomus* larvae provide strong evidence that TBT uptake via sediment ingestion is highly significant and that the feeding behavior of organisms cannot be neglected, when the hazard potential of sediment associated TBT is discussed.

5.5 Acknowledgment

We thank Ingrid Holderegger for the determinations of total and inorganic carbon.

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6

Conclusions and Outlook

Numerous studies have contributed to the present knowledge on the occurrence and behavior of organotin compounds in the aquatic environment, and several different modes of toxic action in aquatic organisms have been reported. In addition, the sources for the two most prominent triorganotin compounds in aquatic environments, i.e., TBT and TPT, are well known. In the case of TBT, primarily areas with frequent shipping activities have to be considered as critical sites. The continual use of TPT as fungicide in agriculture suggests a hazard potential mainly for small aquatic ecosystems adjacent to fields where high amounts of pesticides are used. Predicting possible impacts on organisms and communities that reside in environmental compartments with high organotin concentrations, means that the discrepancy between the measurable total organotin concentrations and the concentrations "seen" by the organisms has to be overcome. Thereby, the bioavailability is of pivotal importance.

The results of this work contribute to a better understanding of the bioavailability of triorganotin compounds and their fate in aquatic ecosystems. The bioconcentration of TPT in *Chironomus riparius* was found to be considerably higher as compared to TBT, due to the absence of an efficient metabolism. As mentioned in Chapter 4, slow metabolism of TPT was also found in other species. The high bioconcentration and the absence of a significant metabolism suggest therefore a higher biomagnification potential for TPT in aquatic food chains than for TBT. In contrast to TPT, the presence of TBT metabolism in many species from low to top trophic levels and the high metabolism rates found in *Chironomus riparius* indicate that a significant biomagnification of TBT is rather unlikely.

Although the concentrations of TBT and of the metabolites DBT and MBT in *Chironomus* larvae could be successfully described with the two-pool model, the fate of the butyltin compounds in the larvae is not yet fully understood. One can speculate that the metabolic activity is highest in the hepatopancreas as found in other arthropods. Another possibility would be that microorganisms in the gut are responsible for the metabolism. The second possibility, however, seems to be less probable, since the *Chironomus* larvae converted TBT into DBT and further to MBT at very high rates, although their guts were more or less empty, when the experiments started. Additional insight into the fate of the butyltins in the *Chironomus* larvae could be gained by exposing *Chironomus* larvae to radiolabeled TBT and studying the distribution of the butyltins in a depuration period, after exposure.

The TBT and TPT bioconcentration experiments in the presence of Aldrich humic acid (AHA) demonstrated that the triorganotin compounds associated with AHA were not bioavailable (Chapter 4). The apparent AHA-water distribution ratios, D_{AHA}, derived from the bioconcentration experiments were generally in good agreement with the the D_{AHA} values determined in dialysis experiments. The bioconcentration-derived D_{AHA} value for TBT at pH 8 was significantly and reproducibly higher than the respective D_{AHA} value obtained with the dialysis experiment. As discussed in Chapter 4, a plausible explanation for the discrepancy is the formation of hydrophobic domains, induced by Ca^{2+} and Mg^{2+}. If this effect was indeed the reason for the observed high D_{AHA} value, a very low bioavailability of TBT in the sediments of alcaline freshwater lakes would be expected.

However, this phenomenon should be systematically investigated, before final conclusions are drawn.

Although it has clearly been demonstrated in Chapter 5 that ingested particles contribute significantly to the total TBT uptake by sediment exposed *Chironomus* larvae, this experiment should be considered as preliminary. To obtain quantitative data on the relevance of the pore water and the particle ingestion pathway, experiments with higher amounts of well defined sediments should be performed, giving the possibility to measure the organotin as well as the DOC concentrations in the sediment pore water. Furthermore, the influence of emptied or filled guts at the beginning of the experiments should be carefully studied.

A.1 Uptake and Elimination of Tributyltin (TBT) by *Chironomus riparius* Larvae at pH 8: Concentrations in Larvae and Water.

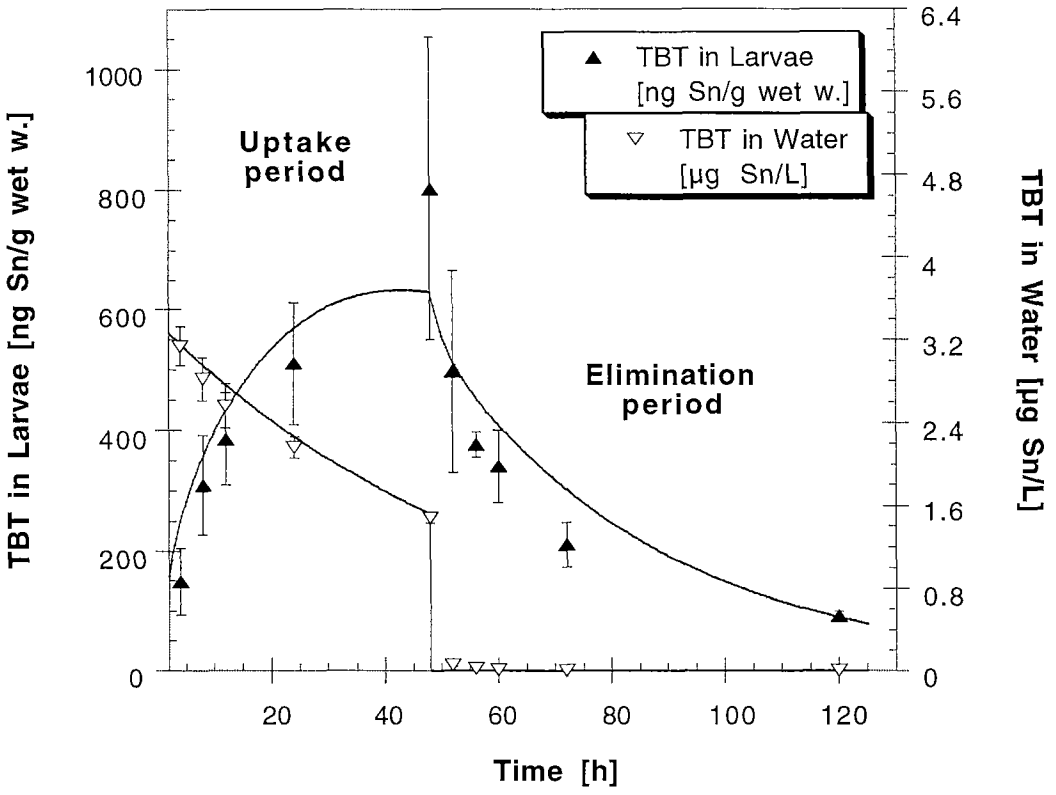

Figure A–1. Time-Course of tributyltin (TBT) concentrations in *Chironomus riparius* larvae and water in an uptake/elimination experiment conducted at pH 8. The concentrations in the larvae and in water were simultaneously fitted according to the model depicted in Figure 4–2 and described by eqs 1 to 10 (Chapter 4). The fitting of the data comprised all TBT, DBT (dibutyltin), and MBT (monobutyltin) concentrations in larvae and water, but only the TBT data are plotted in Figure A–1. Nine parameters were estimated with 63 experimental data points. The data are given as mean ± SD (n = 4).

A.2 Time-Course of Calculated TBT Concentrations in the Two Conceptual Pools of *Chironomus* larvae During 48 hours of Exposure and 72 Hours of Elimination at pH 8.

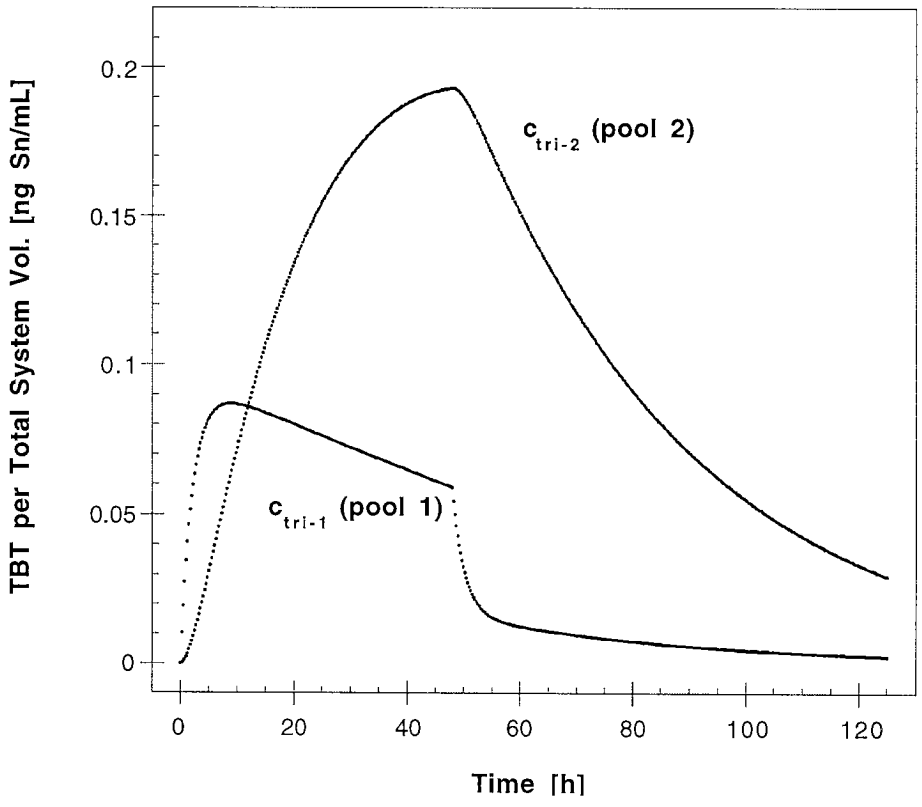

Figure A–2. Time-course of calculated TBT concentrations in the two conceptual pools of *Chironomus* larvae during 48 hours of exposure and 72 hours of elimination. The experiment was conducted at pH 8. The two pools of the model are explained in Figure 4–2 (Chapter 4). The TBT concentration in the first pool reached a maximum few hours after the start of the exposure, whereas the TBT concentration in the second pool increased until the end of the exposure period. The maximum TBT concentration in the second pool was twofold as high as in the first pool. The uptake, excretion and metabolism rate constants are listed in Table 4–1 (Chapter 4) and the rate constants for TBT transfer between the two pools were $k_{trans-12} = 0.117 \pm 0.010$ and $k_{trans-21} = 0.035 \pm 0.003$.

A.3 Time-Course of Calculated DBT (Dibutyltin) Concentrations in the Two Conceptual Pools of *Chironomus* larvae During 48 hours of Exposure and 72 Hours of Elimination at pH 8.

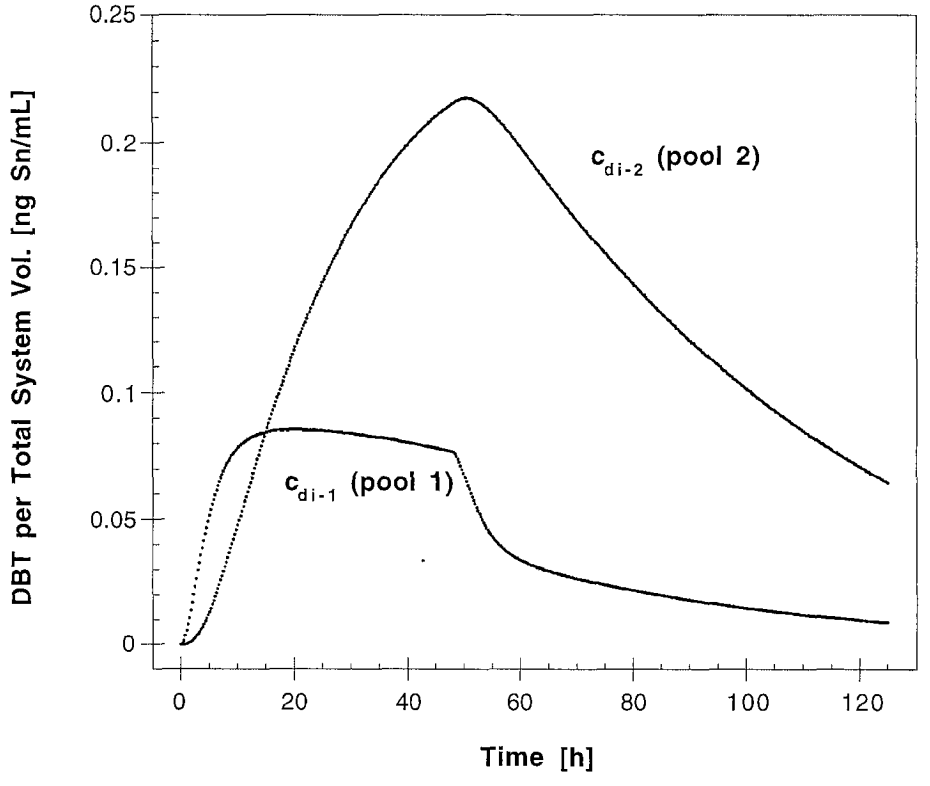

Figure A–3. Time-course of calculated DBT (dibutyltin) concentrations in the two conceptual pools of *Chironomus* larvae during 48 hours of exposure and 72 hours of elimination. The experiment was conducted at pH 8. The two pools of the model are explained in Figure 4–2 (Chapter 4). The DBT concentration in the first pool reached a maximum after about 20 hours and remained at a much lower level than DBT in the second pool. The excretion and metabolism rate constants are listed in Table 4–2 (Chapter 4) and the rate constants for TBT transfer between the two pools were $k_{trans\text{-}12} = 0.117 \pm 0.010$ and $k_{trans\text{-}21} = 0.035 \pm 0.003$.

A.4 Time-Course of Calculated MBT (Monobutyltin) Concentrations in the Two Conceptual Pools of *Chironomus* larvae During 48 hours of Exposure and 72 Hours of Elimination at pH 8.

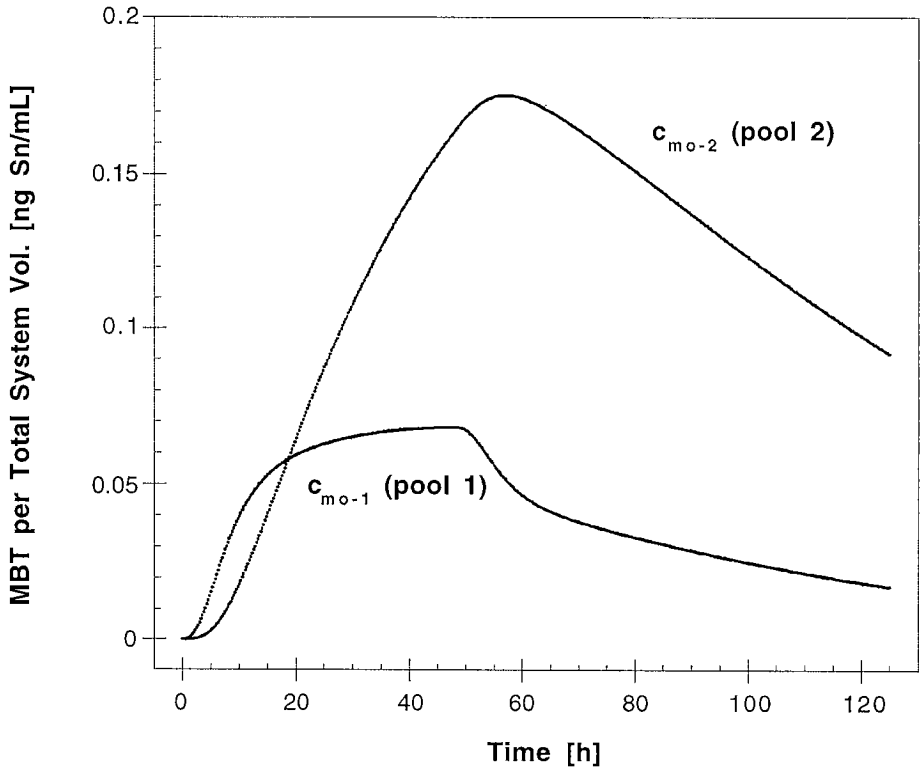

Figure A–4. Time-course of calculated MBT (monobutyltin) concentrations in the two conceptual pools of *Chironomus* larvae during 48 hours of exposure and 72 hours of elimination. The experiment was conducted at pH 8. The two pools of the model are explained in Figure 4–2 (Chapter 4). In contrast to TBT and DBT (see Figures A–2 and A–3), the maximum MBT concentration in the first pool was only achieved after 48 hours, at the end of the exposure period. This is related to the relatively slow rate constant for the debutylation of DBT, as compared to the debutylation of TBT (see Table 4–2 in Chapter 4). The rate constants for MBT transfer between the two pools were $k_{trans-12} = 0.117 \pm 0.010$ and $k_{trans-21} = 0.035 \pm 0.003$.

A.5 Sensitivity of the Calculated Total TBT Concentration in *Chironomus* larvae to Different Adjustable Model Parameters (48 hours of Exposure and 72 Hours of Elimination, pH 8).

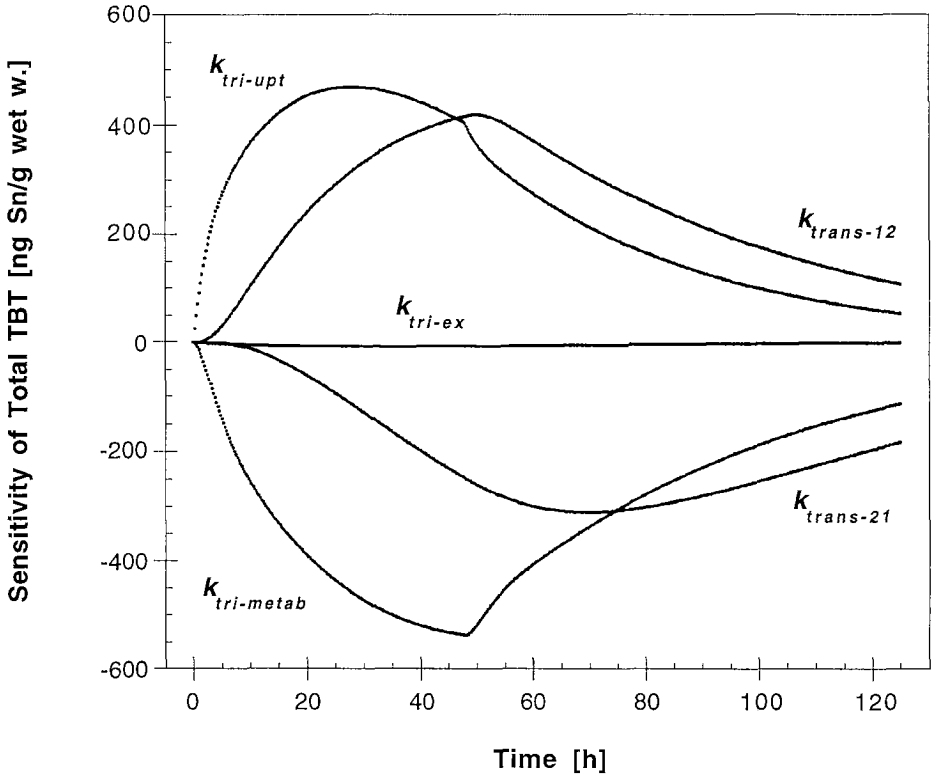

Figure A–5. Sensitivity functions for the adjustable model parameters $k_{tri\text{-}upt}$, $k_{tri\text{-}metab}$, $k_{tri\text{-}ex}$, $k_{trans\text{-}12}$, and $k_{trans\text{-}21}$ during an experiment with 48 hours of exposure to TBT and a 72 hours elimination period. The experiment was conducted at pH 8. The model is explained in Chapter 4 (Figure 4–2). The excretion rate constant, $k_{tri\text{-}ex}$, had a negligible influence on the total TBT concentration in the larvae during the whole experiment. Within the first 24 hours, the uptake rate constant, $k_{tri\text{-}upt}$, had the greatest influence on the total TBT concentration in the larvae. After 24 hours the metabolism rate constant, $k_{tri\text{-}metab}$, became more important. The sensitivity of the total TBT concentration to both transfer rates between the two pools, $k_{trans\text{-}12}$, and $k_{trans\text{-}21}$, reached maxima at the beginning and during the elimination period, respectively. Note that at the end of the elimination period, the transfer rate constant $k_{trans\text{-}21}$ had the greatest influence on the total TBT concentrations in the larvae, i.e., the elimination of TBT was limited by the velocity of TBT transfer from pool 2 to pool 1.

Curriculum Vitae

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