EFFECTS OF BROMINATED FLAME RETARDANTS ON TWO MARINE COPEPOD SPECIES, ACARTIA TONSA AND NITOCRA SPINIPES, AND ON THE ECDYSTEROID-RESPONSIVE DROSOPHILA MELANOGASTER B_{II}-CELL-LINE

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Introduction

Brominated Flame Retardants (BFRs) enter the aquatic environment and have been found in freshwater as well as marine organisms and sediments from many locations around the world.[1] Toxicity studies with BFRs have mainly been performed on mammals. Their acute toxicity seems to be low, but there is concern about various chronic effects. Some BFRs are reproductively, developmentally, immuno- and neurotoxic. They induce certain liver enzymes and act as endocrine disrupters, especially on the thyroid system, but estrogen receptor binding has also been reported.[1-3] At the present time, knowledge about the impact of BFRs on aquatic organisms is very limited. Owing to the lack of data on the ecotoxicity of BFRs, the objective of the present work was to study their effects on growth and development of two ecologically important marine crustacean species, the calanoid copepod *Acartia tonsa* and the harpacticoid copepod *Nitocra spinipes.* To distinguish general pharmacological from endocrine mediated toxic effects, the BFRs were screened *in vitro* for potential ecdysteroid (steroid hormones regulating development and reproduction in arthropods) agonistic/antagonistic effects with the ecdysteroid-responsive *Drosophila melanogaster* B_{II}-cell line. Selected polybrominated diphenyl ethers, PBDEs (BDE-28, -47, -99 and –100), tetrabromobisphenol A (TBBPA), 2,4,6-tribromophenol (TBP) and hexa-bromocyclododecane (HBCD) were tested.

Materials and Methods

TBP and TBBPA were purchased from Sigma-Aldrich Sweden AB, Stockholm, Sweden. Prof. Å. Bergman, Stockholm University, Sweden, kindly provided the PBDEs and HBCD. The *l(2)mbn* (B_{II}) cell line was generously provided by Prof. E. Gateff, University of Mainz, Germany.

A. tonsa larval development tests and *N. spinipes* full life-cycle tests were employed as described earlier[4, 5]. The principle of studying effects on larval development was the same in both test systems. Juvenile copepods pass through six naupliar and five copepodite stages before they reach the adult stage. The easily detectable morphological change from the last nauplius to the first copepodite stage is utilized as the endpoint. At the time, when about 50 % of the control organisms have reached a copepodite stage, the larval development rate (LDR), expressed as the percentage of copepodites among all individuals, was recorded for each replicate of the treated groups and compared with that of the control. Both tests were performed under semi-static conditions with feeding of micro algae (*A. tonsa*) or a suspension based on commercial salmon feed (*N. spinipes*), respectively. In the *A. tonsa* test, eggs were exposed to the test substance in a fully defined saltwater medium (18 ‰ salinity) at 20 ±

0.5 °C under a photoperiod of 12 h light, 12 h dark (low light intensity) for five days. Then, the test was terminated and LDR, hatching success and larval mortality were recorded by using light microscopy. EC-values for the inhibition of larval development in *A. tonsa* were calculated. In the *N. spinipes* test, newly hatched nauplii were exposed at 20 ± 0.5 °C in the dark. LDR and larval mortality were recorded after 6-7 days. The exposure was allowed to continue for another two weeks to study effects on reproduction and population growth (results not shown; publication in prep.). Acute toxicity tests were performed according to Swedish (*N. spinipes*)[6] and international (*A. tonsa*)[7] standard procedures. LC_{50}-values were calculated by probit analysis. Ecdysteroid agonist and antagonist sensitive *Drosophila malanogaster* B_{II}- cell line assays were performed as described previously[8]. This *in vitro* bioassay specifically detects compounds acting as agonists or antagonists at the ecdysteroid receptor complex. The concentration of 20-hydroxyecdysone used in the antagonist version of the bioassay was 5×10^{-8} mol/l. Compounds were tested over the range of 10^{-7} to 10^{-4} mol/l.

Results and Discussion

Acute toxicity tests were employed to determine the concentration ranges to be tested in the (sub) chronic tests. The following 48-h-LC_{50} (mg/l) with corresponding 95 % confidence limits in parenthesis, were determined for *A. tonsa* (compounds ranked according to their toxicity): BDE-28 0.11 (0.093-0.13); TBBPA 0,40 (0.37-0.43); BDE-100 0.52 (0.33-0.87); BDE-99 0.71 (0.34-1.77); TBP 1.50 (1.06-1.83) and BDE-47 2.37 (1.44-21.7). In the sub-chronic test with *A. tonsa*, 5d-EC_{50}-values (mg/l) for the inhibition of the larval development were as low as 0.0072 (0.0006-0.080) for BDE-100; 0.011 (0.006-0.021) for BDE-99; 0.0125 (0.011-0.014) for BDE-47; 0.017 (0.013-0.022) for BDE-28; 0.125 (0.065-0.240) for TBBPA and 0.811 (0.673-0.978) for TBP, respectively. Egg hatching and larval survival were not significantly affected at these concentrations. Acute to (sub)chronic toxicity ratios (AsCR, 48h-LC_{50}/5d-EC_{50}) were 190 for BDE-47, 68 for BDE-100, 63 for BDE-47 and below 10 for the remaining BFRs. Concentration-response curves for the inhibition of the larval development are shown in Figure 1. Except for BDE-100, the data fit the statistical model very well.

The 96-h-LC_{50} (mg/l) determined in the acute toxicity tests with *N. spinipes* were: TBBPA 0.35 (0.30-0.41); BDE-99 > 1; BDE-100 > 1; BDE-47 4.40 (3.70-5.40) and TBP 4.42 (3.62-5.55), respectively. These concentrations are of the same order (TBBPA) or slightly higher than those found in the in the *A. tonsa* acute test. TBP and the BDE congeners 47, 99 and 100 were studied in the *N. spinipes* full life-cycle test. A significant inhibition of the LDR was observed for BDE-47 at concentrations of 0.013 and 0.04 mg/l as well as for BDE-99 at 0.03 and 0.1 mg/l. TBP and BDE-100 did not have any effect on LDR, but larval survival was affected at the highest tested concentrations. Effects on larval mortality and LDR are summarised in Figure 2.

TBP, TBBPA, BDE-28, -47, -99 and –100 were found to be toxic to crustaceans with $LC(EC)_{50}$ values below 1 mg/l. Thus, these BFRs were classified as very toxic to aquatic organisms.

BDE-99 and BDE-100 showed weak ecdysteroid antagonist activity in the B_{II} bioassay at concentrations of $5*10^{-5}$ and 10^{-4} mol/l. TBBPA, TBP, BDE-47 and HBCD showed no agonist or antagonist activity at concentrations up to 10^{-4} mol/l. However, TBP (at 10^{-4} mol/l), TBBPA (at $5*10^{-5}$ and 10^{-4} mol/l) and HBCD (at $2.5\ 10^{-4}$ mol/l) were cytotoxic. TBP, TBBPA and the four tested PBDEs strongly inhibited larval development of *A. tonsa* with 5d-EC_{50}-values ranging from 7 to 800 µg/l. Moreover, BDE-47 and –99 showed an inhibitory effect on the development of *N. spinipes* larvae. In the *A. tonsa* assay, very high AsCRs were found for BDE-47, -99, and-100, which could be regarded as an indication of different modes of toxic action in the acute and the sub-chronic tests, respectively. This is consistent with various specifically toxic effects of BFRs observed in vertebrates.[1-3] A link between endocrine disruption as well as specific toxicity in general and the inhibition of larval development in copepods at sublethal concentrations (AsCR > 10) was discussed previously[4]. Both moulting and

metamorphosis in crustaceans are hormonally regulated by ecdysteroids. Moreover, the sesquiterpenoid methyl farnesoate (similar to the juvenile hormones [JH] known from insects) also regulates metamorphosis in crustaceans[9]. The inhibition of the development of juvenile copepods might be a result of an interaction with the ecdysteroid or JH systems. This is further supported by the ecdysteroid antagonistic activity found for BDE-99 and BDE-100, which were the most potent inhibitors in the *A. tonsa* test. Thus, at least these two PBDEs have to be considered as potential endocrine disrupters in invertebrates.

Further research on invertebrates is needed to elucidate the toxic mode of action of compounds, for which whole-organism testing or field studies suggest endocrine disruption. The combination of *in vitro* screening methods and (sub)chronic copepod bioassays can provide useful information in this context.

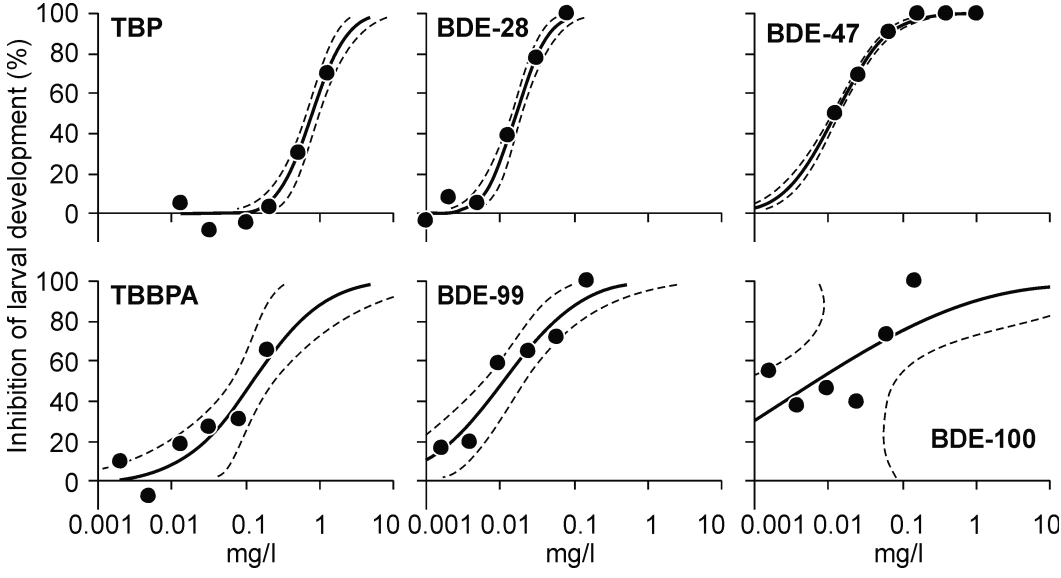

Figure 1. Experimental values and estimated concentration-response curves with corresponding 95 % confidence limits (dotted lines) for the inhibition of the larval development of *A. tonsa* exposed to TBP, TBBPA, BDE-28, -47, -99 and-100, respectively, at 20 ± 0.5 °C for 5 days.

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References

1. de Wit CA (2002) Chemosphere.46, 583

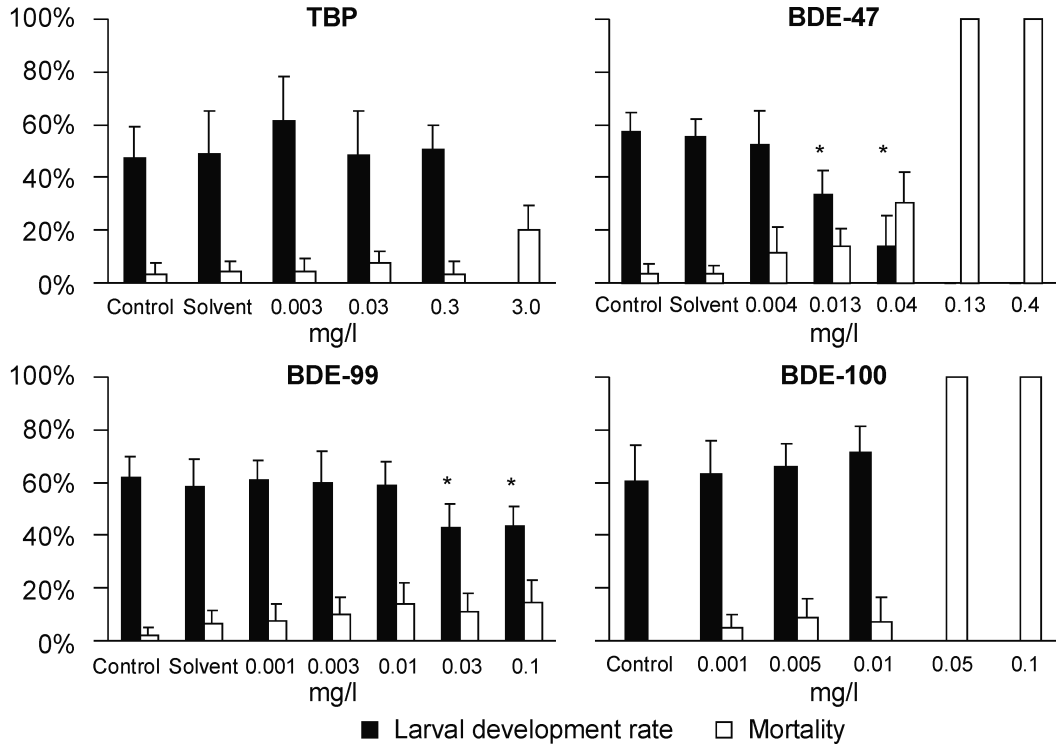

Figure 2. Larval development rates and mortality of juvenile *N. spinipes* exposed to TBP, BDE-47, BDE-99 and BDE-100, respectively, for 7 days. Error bars indicate 95 % confidence intervals of means. Asterisks denote a significant difference to the control ($p < 0.05$).

2. Darnerud PO, Eriksen GS, Johannesson T, Larsen PB and Viluksela M (2001) Environ Health Persp.109 Suppl, 49
3. Meerts IATM, Letcher RJ, Hoving S, Marsh G, Bergman A, Lemmen JG, van der Burg B, Brouwer A (2001) Environ Health Persp.109, 399.
4. Andersen HR, Wollenberger L, Halling-Sørensen B, Kusk KO (2001) Environ Toxicol Chem.20, 2821
5. Breitholtz M.and Bengtsson B-E. (2001) Mar Pollut Bull.42, 879
6. SIS (1991) Swedish Standard SS 02 81 06 Determination of acute lethal toxicity of chemical substances and effluents to *Nitocra spinipes* Boeck - Static procedure. SIS - Stockholm, Sweden.
7. ISO 14669, Water Quality - Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea)
8. Dinan L, Bourne P, Whiting P, Dhadialla TS, Hutchinson TH (2001) Environ Toxicol Chem.20, 2038
9. Chang E.S. (1993) Annu Rev Entomol.38, 161