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Imposex Induction in the Mud Snail, *Ilyanassa obsoleta* by Three Tin Compounds

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Pseudohermaphroditism or imposex was first described in the neogastropod, *Nucella lapillus* by Blaber (1970). This morphological condition is characterized by the development and superimposition of non-functional male accessory sex organs (i.e. vas deferens and/or a penis) on female or juvenile gastropods (Jenner, 1978; Gibbs et al. 1990; Gibbs et al. 1987; Oehlmann et al. 1996). In extreme cases, females can become sterile resulting in a localized extinction of exposed populations (Bryan et al. 1986). The occurrence of imposex is directly linked to low levels of organotins in the environment (Gibbs et al. 1987). Organotins, such as tributyltin (TBT), dibutyltin (DBT) and triphenyltin (TPT) enter the environment through their use in antifouling paints, as molluscides, and as catalytic agents in the formulation of various agricultural and industrial products including many plastics (Tas et al. 1996). Exposure of gastropods to these organotins, even at very low concentrations (as low as 0.5 ng/L Sn) can induce imposex (Oberdörster et al., 1998). The environmental effects of both TBT and TPT have been extensively studied; however only a few studies have examined the effects of other tin compounds (Mattheissen and Gibbs, 1998). Organotins other than TBT or TPT serve a limited function as anti-fouling agents but have been used extensively in the production of PVC plastics agricultural products and a variety of manufacturing and industrial applications (Fent, 1996, White et al. 1999). Their presence in the environment reportedly occurs at low concentrations ranging from 0.002-0.8 ng/L Sn however, they do remain in the sediments for many years and have the potential to bioaccumulate (Ramaswamy et al. 2004). The goal of this study was to determine whether exposure to three industrial tin compounds: trioctyltin chloride (TOT), diocytltin chloride (DOT), and tin tetrachloride (TTCl) cause imposex induction in the common mud snail *Ilyanassa obsoleta* and to compare their biological impact to that elicited by tributyltin chloride.

MATERIALS AND METHODS

Mud snails (*Ilyanassa obsoleta* (Say)) were collected on the mud flats of Bird Shoals in the Rachael Carson Estuarine Research Reserve near Beaufort, NC.

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This location has a background imposex rate of < 7% (Oberdörster et al. 1998). Approximately 2200 snails ranging in size from 12.9 x 8.4 mm to 17.9 x11.1 mm (length x width of shells) were collected. The snails were acclimated to laboratory conditions using a 14:10 light/dark cycle for 2 weeks prior to initiating the study.

Injection studies were carried out using forty snails placed in 20 cm glass finger bowls containing 250 mL (approximately 1 cm deep) of well-aerated aged filtered seawater. Snails received 20 ng tributyltin chloride, trioctyltin chloride or tin tetrachloride in an injection volume of 5 μl every other day for 7 or 14 days. The tin compounds were injected into the dorsal head region of the snail using a 25 μl tuberculin syringe. The water was changed daily and the snails were fed 1-2 grams of fish on day 0 and day 10 of the study. Twenty snails were sacrificed on days 7 and 14 in each exposure group. The shells were removed and the snails sexed based on the presence or absence of a penis and vas deferens, the presence of an egg capsule gland, and appearance of the gonads.

Aqueous exposures were carried out by arbitrarily dividing the snails into groups of 30 individuals and placing them in 750 ml of aged filtered sea water taken from the Beaufort Inlet. Analysis of the sea water revealed no detectable organotin compounds. One of four compounds, trioctyltin chloride (TOT), diocytltin chloride (DOT), tin tetrachloride (TTCl) or tributyltin chloride (TBT), was dissolved in 100% ethanol and added to the containers at 5, 10, 20, or 50 ng/L. An additional group received a comparable amount of 100% ethanol as a solvent control. Each exposure was repeated in quadruplicate. Solutions were renewed daily for 45 days, and snails were fed 1-2 grams of fish at 10 day intervals. The temperature remained constant at 19°C with a 14/10 hour light/dark cycle.

Imposex induction was determined by calculating the percentage of females bearing a penis or penis bud. The severity of the morphological changes in the aqueous exposed snails was calculated using the Relative Penis Size Index (RPSI $= (FPL^3/cMPL^3)*100$). [FPL = Female Penis Length; cMPL = control Male Penis Length] (Gibbs et al. 1987). Statistical analysis was carried out using SYSTAT version 8.0 for IBM for ANOVA, Excel 97 for Chi-squared analysis and Good Fit Analysis. Ranking of induction was determined using a contingency analysis. Contingency analysis is the equivalent of ANOVA using frequencies and generates significant and non-significant subsets Sokal and Rolhf (1981).

RESULTS AND DISCUSSION

Each of the tin compounds tested in this study induced imposex in *I. obsoleta*. TBT was by far the most potent compound tested. Injection of the compounds resulted in a 35% induction by TBT, 15% induction by TOT and 18% induction by TTCl after 14 days (Figure 1). These levels were statistically different from the control group ($p \leq 0.001$) (See Figure 1). An increase in imposex was

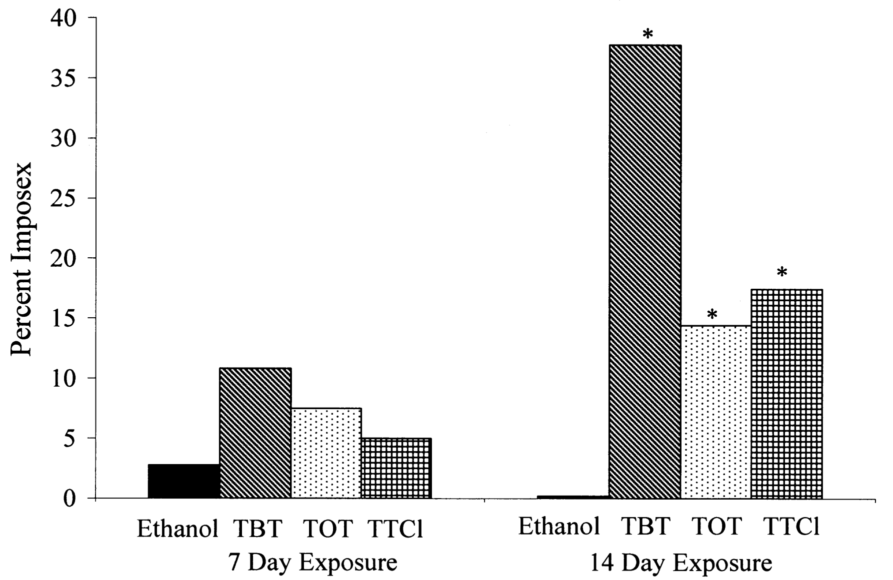

Figure 1. Imposex induction following injection of three tin compounds. Mud snails were injected with 20 ng of TBT, TOT or TTCl every other day for 14 days. On days 7 (A) and 14 (B), twenty snails were removed from their shells and the percentage of females that developed a penis or penis bud was recorded (* $p \leq 0.001$).

observed by 7 days but, this level was not statistically greater than the controls. Aqueous exposure of the snails to TBT resulted in a 35-40% induction of imposex development compared to 25-27% for TOT, 18-19% for DOT and 21-23% for TTCl (Figure 2). Analysis of the frequency data by contingency analysis (RXC and Good Fit Analysis; Sokal and Rohlf, 1981) indicated that all of the compounds have at least one highly significant result ($p \leq 0.001$) indicating that all the compounds induce imposex in the exposed snails (See Table 1).

Induction of imposex occurred in a dose dependent manner in the aqueous exposures through the 50 ng/L concentration and followed the pattern TBT >>TOT≅DOT≅TTCl. Studies employing a concentration of 100 ng/L of TOT or TTCl indicated a lower increase in imposex induction (data not shown). This lower induction was most likely due to a limited solubility of the compounds in sea water at the higher concentration and thus a lower uptake by the organisms (Eng et al. 1988).

The induction of imposex in both the injection and aqueous exposure studies by TTCl was surprising. Prior to this report, exposure of gastropods to inorganic tin has not been reported to cause imposex. It is possible that during the aqueous

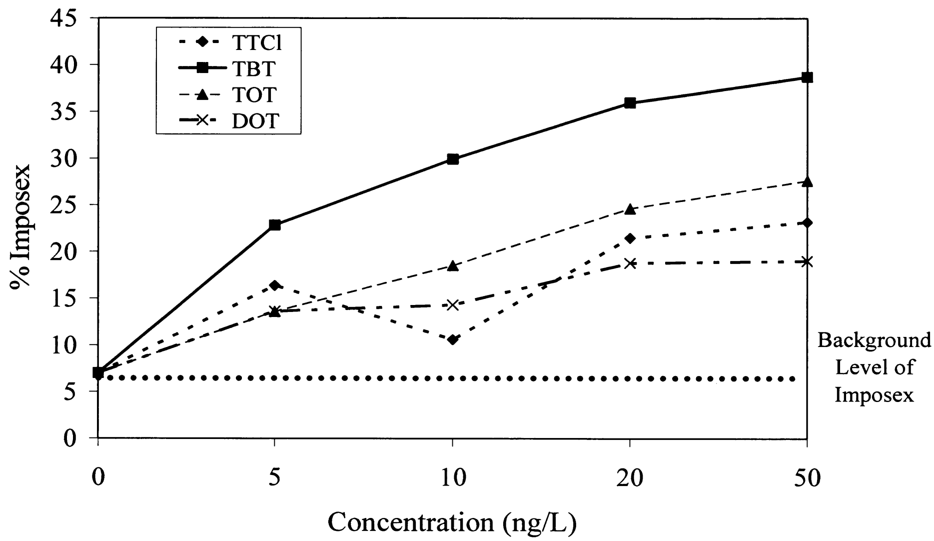

Figure 2. Imposex induction in response to four tin compounds. Mud snails were exposed to various concentrations of TBT, TOT, DOT and TTCl for 45 days. The percentage of females that developed a penis or penis bud was recorded at the end of the exposure period.

exposures, sulfate reducing bacteria residing in the organic lawn on their shells were responsible for methylating the TTCl (White et al. 1999). Humic substances on the shells could also facilitate the methylation of these compounds. Methylation of tin increases the volatility, lipid solubility and absorptivity of the compound thereby influencing its toxicity (Cooney, 1988). Uptake of this methylated tin could therefore result in a low level of imposex development. However, imposex induction via TTCl exposure was also observed in snails injected with the compound (Figure 1). This indicates that methylation of the tin compound by resident lawn bacteria may not be responsible for this process. Further studies are necessary to verify this supposition and determine the precise mechanism(s) of induction.

Two morphological changes seen during imposex development are the production of a penis bud and/or the growth of the penis shaft (Oberdorster and McClellan-Green, 2000). In this study, the penis bud as it developed in response to octyltin exposure was a small bump or node of 0.1-0.2 mm in diameter near the right tentacle. Penis shafts, as long 10 mm, were observed in snails exposed to the higher aqueous concentrations of TBT. Thus, the development of the penis shaft was not as dramatic nor as elongated following exposure to the octyltins or tin tetrachloride as with the more potent TBT compound.

The growth of these accessory sex organs have been used as an indicator of the severity of imposex development. The Relative Penis Size Index (RPSI) was

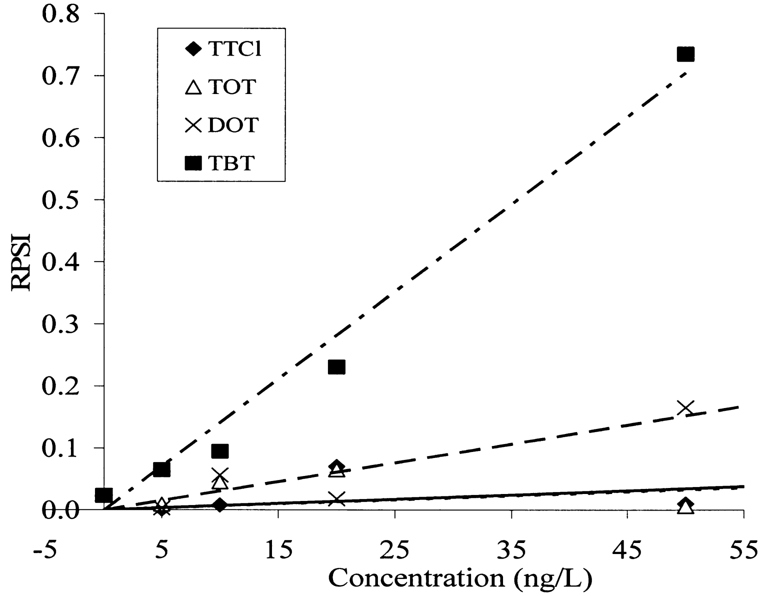

Figure 3. The Relative Penis Size Index (RPSI) was calculated for each of the exposure groups. The size of each penis or penis bud was recorded for snails exposed to TBT, TOT, DOT, TTC and the control.

previously employed by Gibbs et al. (1987) to describe the potential for various organotins to function as endocrine disruptors. Female snails exposed to TBT exhibited a dramatic and dose dependent increase in RPSI to a maximum of 0.74 (Figure 3). The effect observed in animals exposed to TOT, DOT, and TTCl was minimal with an RPSI $\leq$ 0.10 and followed the pattern TBT>>DOT>TOT≅TTCl. This indicates that exposure to octyltin and tin tetrachloride are sufficient for the initiation of the growth of the accessory sex organs, but they do not appear to be as effective as TBT at stimulating neuronal and/or hormonal factors necessary for supporting full development of imposex characteristics.

It is possible that a lower uptake of the octyltins or tin tetrachloride by snails from an aqueous media is responsible for this differential effect. As a rule of thumb, the toxicity of organotins increases with the number of organic groups such that the tri-substituted compounds, e.g. tributyltin, will have a higher toxicity than the di- or mono- substituted compounds (Matthiessen and Gibbs, 1998). This was evident in our study. However, it is also true that the polarity of a compound plays a key role in the uptake and accumulation of organotins and strongly influences their toxicity. An increase in the alkyl chain length of organotins normally lowers the toxicity of the compound. For example, triethyltin acetate ($Et_3SnOCOMe$) has relatively short carbon chains and is considered the most toxic of the organotins to mammals (Rat LD_{50} = 4mg/kg) (Omae, 2003).

Alternatively, longer chain compounds such as the octyltin derivatives (8-carbon chains) are usually considered non-toxic to organisms hence, their use as stabilizers in polyvinyl chloride (PVC) materials. This relationship is supported through the results of this study.

Table 1. Statistical comparison of the data was conducted using pooled data if the individual trials were statistically similar (non significant subsets) based upon contingency analysis. I = Imposex; N= Normal. Expected ratio is the ratio of I to N in the group to be compared. CONT is the sea water control. TBT is the TBT treatment. G is the G statistic, in this case generated by the Good Fit statistical test (= chi square value with 1 degree of freedom). p is the probability of obtaining a larger G value by chance.

CONDITION	FEMALES I/N	EXPECTED (CONT or TBT)	G	p
TBT 5+10	25:69	7:64(C)	21.2	<0.001
TBT 20+50	36:59	7:64	52.9	<0.001
TOT 5+10	17:94	7:64 (C)	3.2	NS
TOT 20+50	26:72	7:64	22.1	<0.001
TOT 5+10	17:94	25:69 (TBT)	8.1	0.005
TOT 20+50	26:72	36:59	5.7	0.025
DOT 5+10	14:86	7:64(C)	1.7	NS
DOT 20+50	19:85	7:64	22.1	<0.001
DOT 5+10	14:86	25:69 (TBT)	9.271	<0.005
DOT 20+50	19:85	36:59	18.9	<0.001
TTCl 5+10	17:105	7:64(C)	2.0	NS
TTCl 20+50	27:90	7:64	17.4	<0.001
TTCl 5+10	17:105	25:69 (TBT)	11.4	<0.001
TTCl 20+50	27:90	36:59	11.7	<0.001

Only 10-30% of organometals in aquatic environments occur in solution while the remaining 70-90% is found in the solid-phase fractions (White et al. 1999). Soils, sediments and particulate matter act as a sink for organotin compounds where their half- life ranges from 1-9 years. The occurrence of octyltin compounds in the environment has not been widely examined. Ramaswamy et al (2003) reported water concentrations of octyltins at 17 locations near Uwakai, Japan. These concentrations ranged from 0.041 to 0.795 ng/L for MOT, 0.002 to 0.262 ng/L for DOT and 0.002 to 0.012 ng/L for TOT. The corresponding sediment concentrations at these locations ranged from 0.28 to 1.9 ng/g for MOT, 0.22 to 0.59 ng.g for DOT and 0.12 to 44.6 ng/g for TOT. Interestingly, octyltin residues were not detected in the oyster tissues analyzed in their study. They hypothesized that the lack of accumulation was due to the octyltins binding to humic substance in the sediments. The presence of humic material, except for minimal amounts resident on the snail shells, was not an issue in our study. Hence, further research on the fate of octyltins in the environment is necessary to determine whether they are bioavailable to gastropods or whether they are mechanistically incapable of eliciting imposex induction.

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