Integrated Approaches to Testing and Assessment Strategy: Use of an Adverse Outcome Pathway to Inform Risk Assessment

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Purpose

- To illustrate an approach to informing risk assessment through use of an adverse outcome pathway using triclosan as an example.

- For illustration purposes only; not a risk assessment

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Background on Triclosan as AOP case study

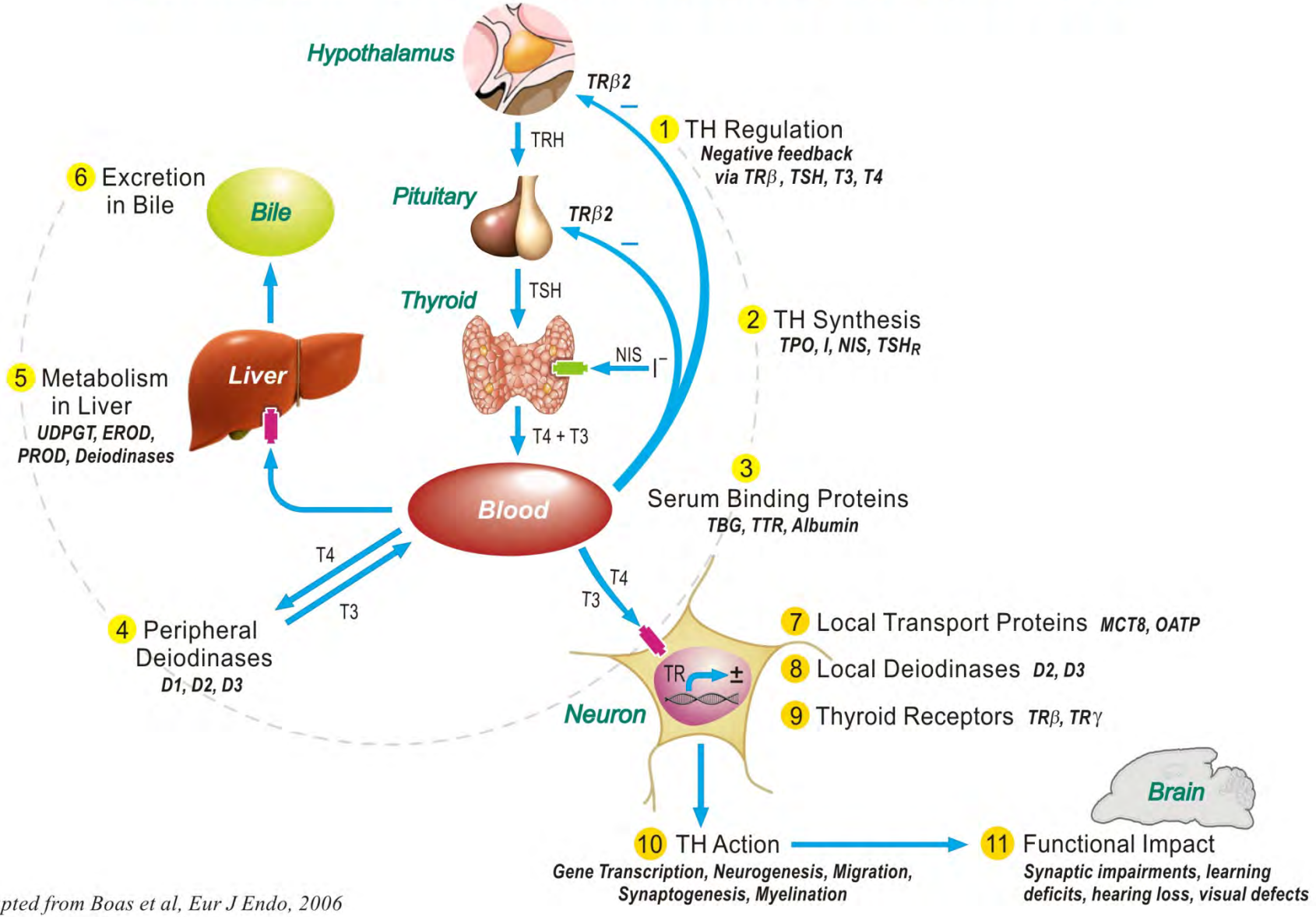
• Antimicrobial pesticide re-assessed by the Agency in 2008 with a wide range of materials preservative uses regulated by EPA. Uses also exist for triclosan under FDA regulation.

• EPA has a full data set of submitted mammalian toxicity studies that investigated general toxicity, as well as studies investigating developmental, reproductive, mutagenic, and carcinogenic properties of triclosan.

• There were also published studies suggesting a possible effect of triclosan on the thyroid hormone endocrine system.

•Alterations of thyroid hormone levels is of particular concern as disruption of circulating concentrations of thyroid hormones have been associated with adverse outcomes in humans, including altered neurodevelopment

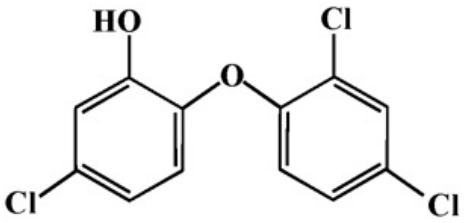
Basic Thyroid Toxicology 101



Adapted from Boas et al, Eur J Endo, 2006

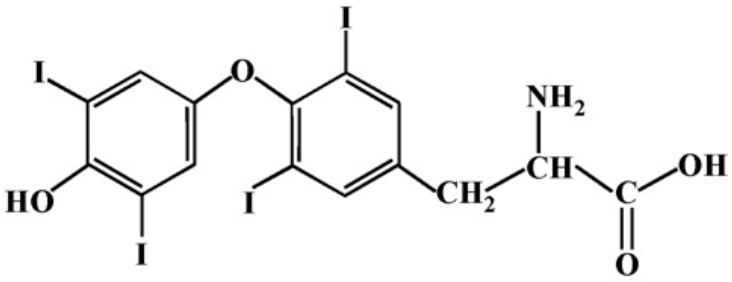
Triclosan is structurally similar to thyroxine

Triclosan



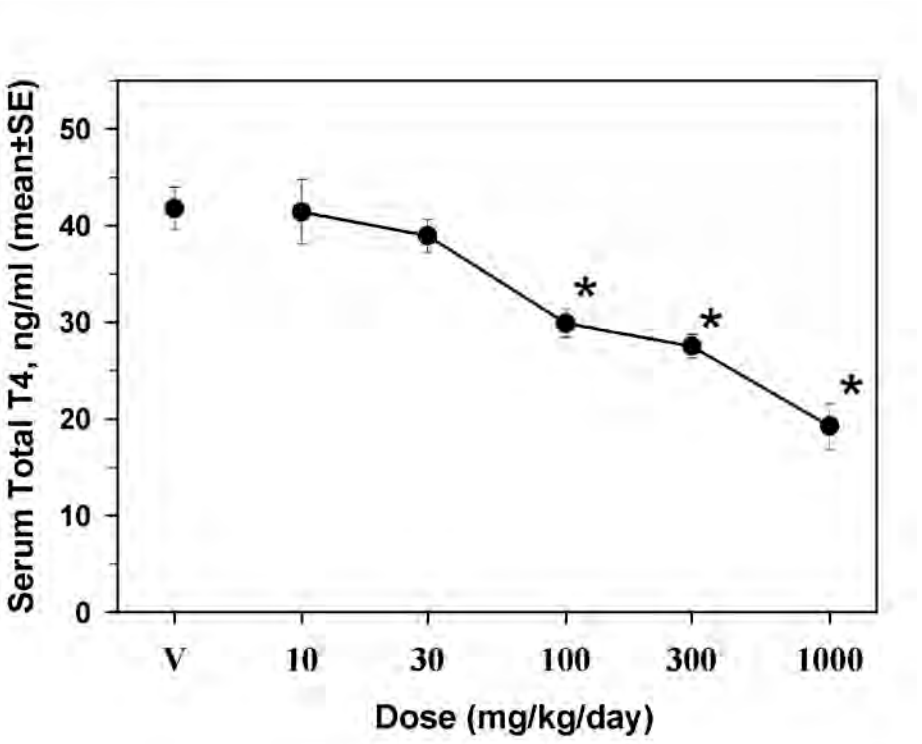
Thyroxine

(T4)



There is evidence of possible thyroid hormone disruption by triclosan

- 4-day oral triclosan exposure in adult male rats
- Decreased serum thyroxine (T4) and increased liver weight
- Not indicative of any particular molecular initiating event, but evidence of a downstream effect



Crofton et al. 2007

Triclosan AOP questions

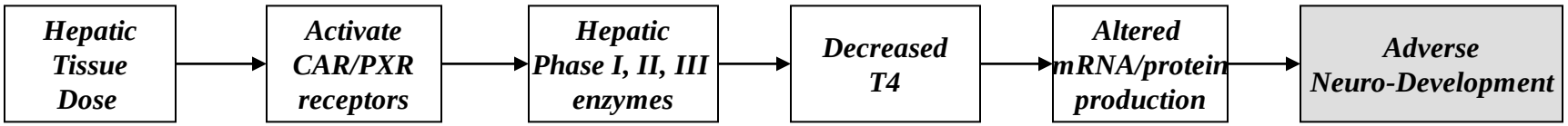
These findings raised several questions, including:

- Can an AOP be identified for triclosan?
- What is the molecular initiating event in the AOP? In other words, what starts all of this?
- Do humans respond in the same manner as experimental animals to this effect of triclosan? Is the AOP the same between species, assuming one can be identified?
- Is there sensitivity of different life stages to this effect?
- What are the implications for how we would do the risk assessment for triclosan?

How do we go about demonstrating if there is an AOP for triclosan's thyroid hormone effects?

- A combination of in vivo/ in vitro/ in silico studies to confirm or deny the series of events in the proposed AOP.

What do we think the AOP is?



1. Activation of hepatic nuclear receptors (CAR/PXR)
2. Upregulation of hepatic Phase I, II, and III metabolism
   - Hepatic UGTs and SULTs
3. Catabolism of thyroid hormones
4. Increased biliary elimination of thyroid hormones
5. Decreased circulating thyroid hormones
6. *Decreased tissues levels of thyroid hormones*
7. *Altered tissue specific mRNA and protein production*
8. *Altered neurodevelopment*

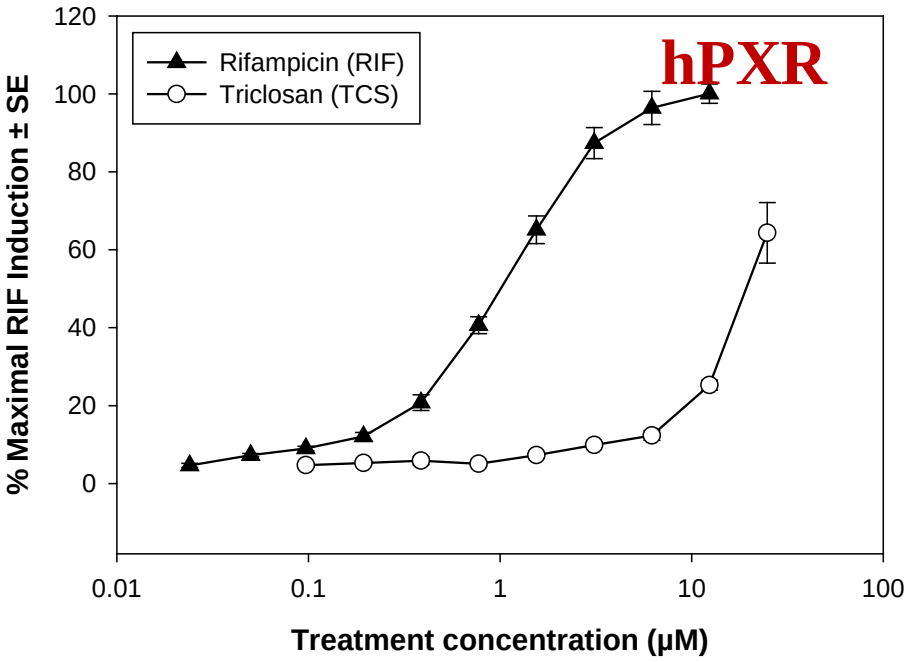
Does triclosan activate PXR and/or CAR?

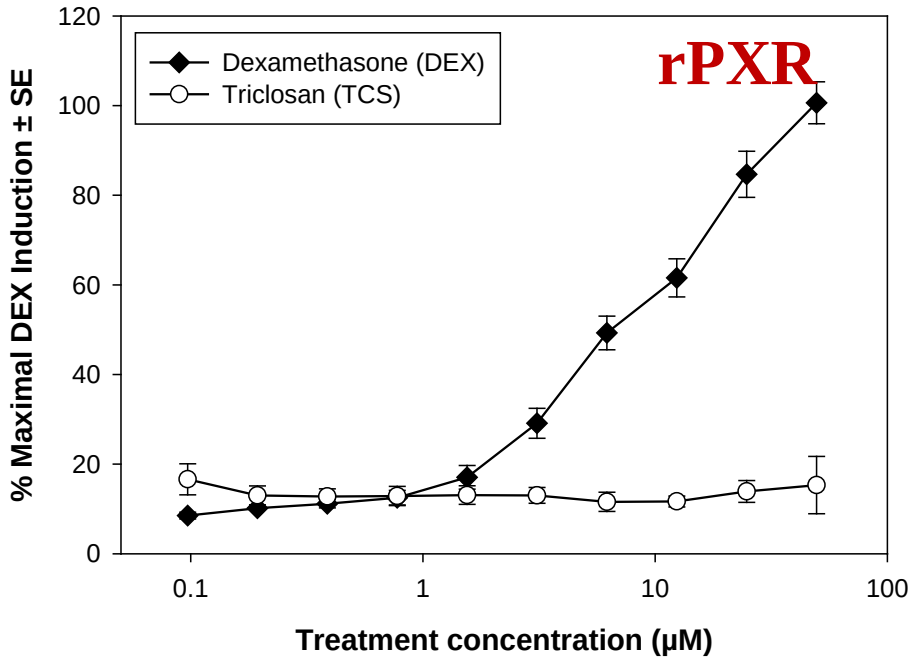
- No PXR activation observed at 2µM triclosan in multiplexed reporter gene assays run by Attagene.
- Reporter gene assays for PXR run at the NCGC for both rat and human PXR also negative.
- Cytotoxicity a possible limiting factor in these assays.

- Triclosan was active in a biochemical PXR binding assay for run at Novascreen which showed an AC_{50} of 13 mM.
- Increases in target genes for PXR and CAR nuclear receptors in the quantitative nuclease protection assay (qNPA) run by CellzDirect on primary human hepatocytes also observed.
- Data suggest triclosan may have PXR and possibly CAR agonist activity but at concentrations concurrent with cytotoxicity in the *in vitro* cellular assays.

Nuclear Receptor Activity

Triclosan activates human PXR but not rat PXR

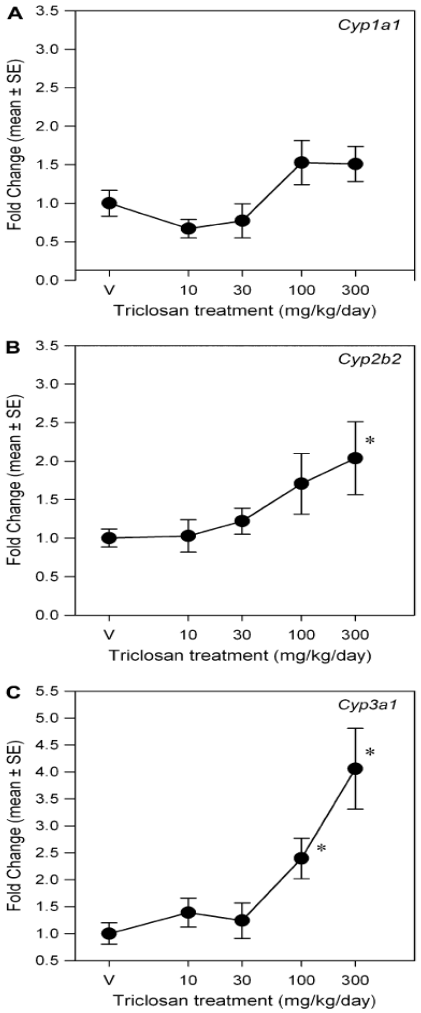




Next AOP step: Does triclosan induce hepatic enzymes?

- Study by Paul et al 2010
- In this study, female Long-Evans rats aged 27-29 days of age were exposed to oral doses of triclosan for four days at dose levels of 0, 10, 30, 100, 300, and 1000 mg/kg/day

Paul et al. study results

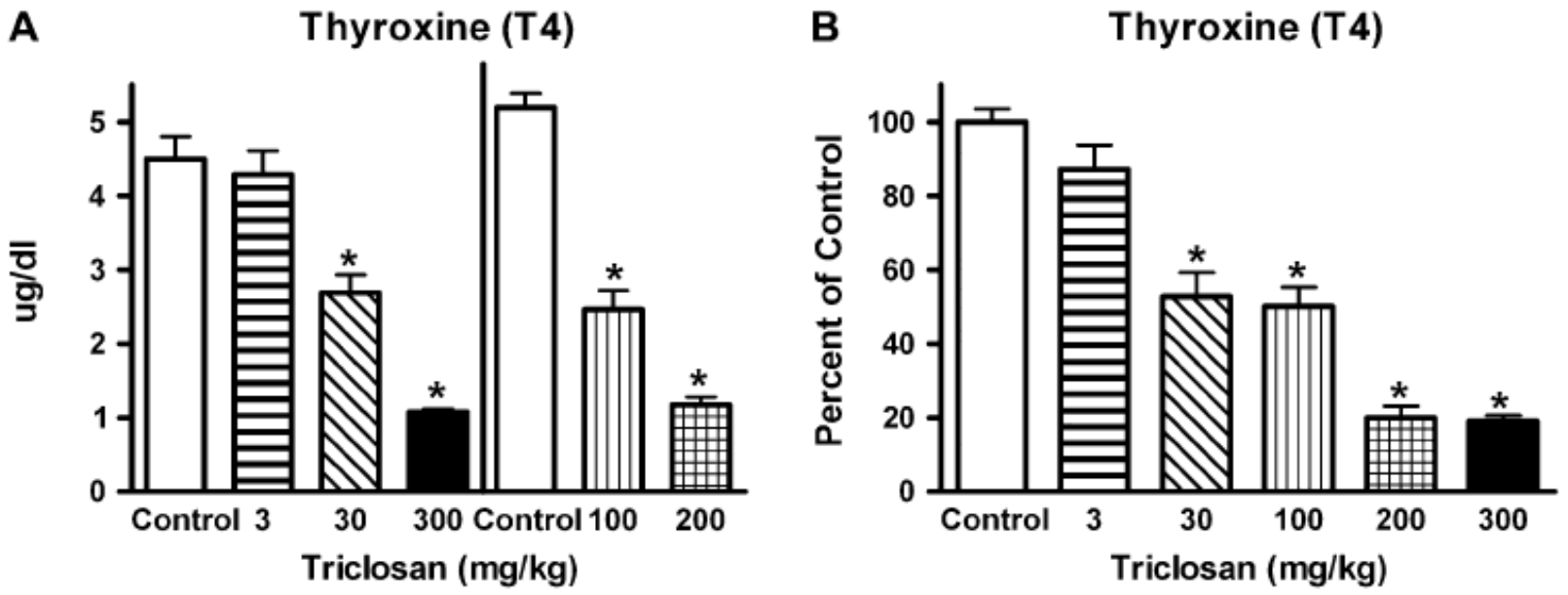


Paul et al study results cont.

- “Cyp1a1 mRNA expression was not altered by triclosan treatment, but Cyp 2b1/2 and Cyp 3a1/23 were increased in a dose-dependent manner. Triclosan increased hepatic PROD activity, but not EROD activity. T4-Glucuronidation activity towards triclosan also increased in a dose-dependent manner, and isoform-specific increases in UGT and SULT mRNA expression were also observed.”

Next step: does triclosan affect thyroid hormone levels?

Zorilla et al 2008: 31 day exposure to triclosan in juvenile male rats



AOP conclusions

- Triclosan has been shown to activate the hepatic PXR receptor.
- Triclosan has been shown to induce levels of hepatic enzyme mRNA that lead to increased activity of glucuronyltransferase and sulfatase.
- This in turn leads to increased elimination of triclosan from the body, and decreased circulating T4/T3.

Importance of the thyroid AOP

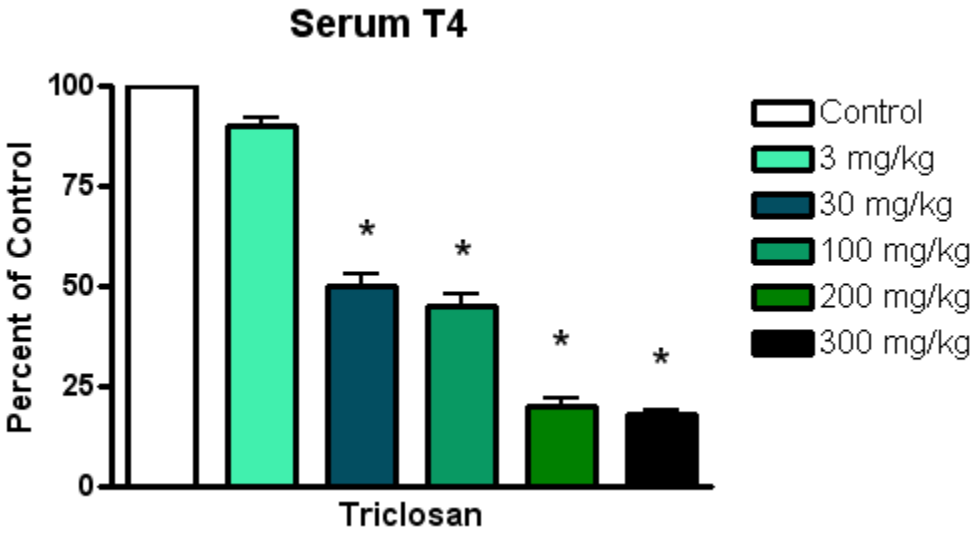
- The developing nervous system is dependent upon adequate amounts of thyroid hormones, and significant neurological damage can occur when the deficiency is present during brain development (Obregon, 2007).

- Deficiencies of thyroid hormone at birth can result in deficiencies in mental and neurologic outcome at 2 and 5 years of age (van Wassenaer, 2002).
- Thus, it is important to know if triclosan has an effect on thyroid hormone levels in early life stages

Studies examining the effect of triclosan in early life stages

- Zorilla et al (2008) used the EPA Endocrine Disruptor's Screening Program (EDSP) Tier 1 Pubertal Male Protocol (31 day oral exposure from PND 23 to 53) to examine effects of oral triclosan exposure in weanling male rats.

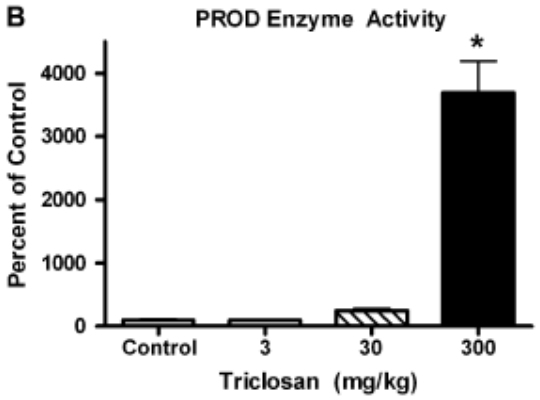
Zorilla et al, cont.

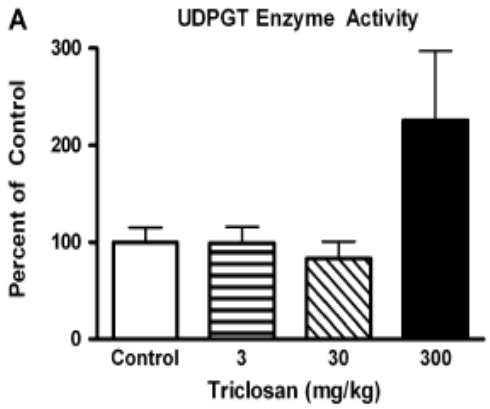


Suppression of serum T4 at 30 mg/kg (LOAEL) and above. No effect on TSH levels.

Zorilla et al, cont.

- Liver weight was significantly increased at 100 mg/kg and above in this study. Liver enzyme activity was also significantly increased.

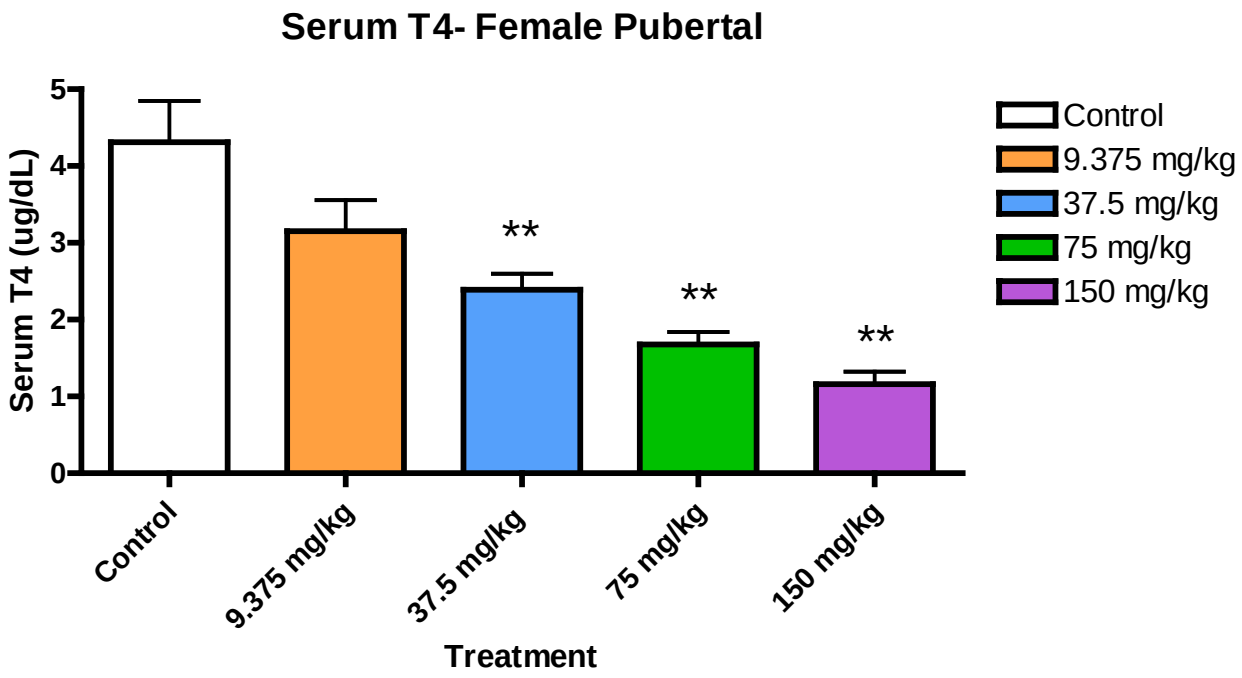




Stoker at al. (2010)

- Evaluated exposure to triclosan in weanling female rats using the EDSP Tier 1 female pubertal protocol (21 day oral exposure from PND 23 to 53) at doses of 0, 9.375, 37.5, 75, and 150 mg/kg/day.

Stoker et al. 2010 cont.



Paul et al. (2010)

- Looked at whether perinatal triclosan exposure would alter circulating thyroid hormone levels in pups during early postnatal development and in dams at the conclusion of lactation.

- Dams received oral doses of 0, 30, 100, and 300 mg/kg/day from gestation day 6 through post-natal day 21.
- Pups were sacrificed on PND 4, 14, and 21 for examination of serum thyroxine.
- Dams were sacrificed on PND 22

Paul et al. (2010) cont.

- Perinatal exposure to triclosan in this study had no effect on gestation length, litter size, viability, or sex ratio.
- Serum thyroxine was decreased in maternal animals on GD20 and PND 22 by approximately 30% at 300 mg/kg.
- Serum thyroxine in pups was decreased 27% on PND4 at 300 mg/kg triclosan, but not on PND14 or PND21 at any dose level.

Summary of early life stage data

Studies provide dose-response data for suppression of T4 across several life stages and durations of exposure.

A similar dose-response was observed between males and females given that the exposures were different (21 vs. 31 days).

Within rat strains, the fetus or weanling rat did not appear to be more sensitive to the effects of triclosan on serum thyroxine, as compared to the dam or older animals.

Areas of Uncertainty

- Understanding of the differences in thyroid hormone regulation and pharmacokinetic differences in rats versus humans are areas that influence the conclusion whether an adverse effect would occur in humans from exposure to triclosan.

Species differences in thyroid regulation (inter-species extrapolation)

- Intended to account for differences in response between animals and humans

- Compared to humans, where TBG is the major T4 binding protein, most T4 in adult rat serum is bound to albumin and transthyretin (TTR); rats do not possess TBG after PND 35.

Species differences in thyroid regulation (inter-species extrapolation), cont.

- Binding affinity of T4 for TBG is more than 100-fold greater than that of albumin or TTR.

- Differences in binding and T4 clearance suggests that rats may be more sensitive to perturbations of circulating T4 than humans and is an important consideration in application of the AOP to human populations.

What does all of this mean for our risk assessment?

- In the 2008 triclosan RED, we chose a NOAEL value of 30 mg/kg/day from a chronic toxicity study based on changes in clinical signs of toxicity for our oral risk assessments.
- This is a non –specific effect and we end up using our standard uncertainty factors (10x) for inter- and intra-species variation/extrapolation.

What does all of this mean for our risk assessment?

- Knowledge of the AOP can provide useful information in the dose–response assessment for a substance. If the quantitative relationships are understood for precursor events or key events within a causal path leading to a disease or adverse outcome, the key event(s) can serve as the basis of for the dose response analysis
- Knowledge of the AOP and its application across mammalian species can also help us address inter- and intra-species uncertainties.

Relevance of the AOP to risk assessment

- In the triclosan case, instead of a general effect such as body weight which we have used in our existing assessment, use of an endpoint based on the AOP allows us to regulate on an effect more relevant for protection of human health.

Relevance of the AOP to risk assessment

- Instead of using a NOAEL based on changes in body weight, we can calculate a benchmark dose, setting the benchmark response at 20% (i.e. using the benchmark of a 20% decrease in T4 as the relevant effect and magnitude of effect).

Relevance of the AOP to risk assessment

- The 20% BMR was chosen based on the clinical literature indicating perceived toxicological significance of a change in the measured endpoint (decreased T4 concentration), and general clinical knowledge about adverse outcomes associated with decreases in circulating thyroid hormones.

Relevance of the AOP to risk assessment

Less than 1% of T4 in humans is freely circulating and available for destruction by liver enzymes, resulting in humans having a greater resistance than the rat model to thyroid toxicity, which occurs secondary to liver enzyme activation.

Thus, it is likely that humans will be less responsive to triclosan-induced changes in serum T4 levels.

Relevance of the AOP to risk assessment

- Based on knowledge of the AOP and species differences in thyroid regulation, we can reduce the inter-species factor from 10x to 3x. The intra-species factor remains at 10x to account for differences in response among the human population.

End of case study