Appendix D:

Aquatic Ecotoxicology Studies

Appendix D: Aquatic Ecotoxicology Studies

Table D.1: Fish studies evaluated for occurrence of NMDR in thyroid.

Reference	Chemical	Species
Bradford et al. (2005)	Sodium perchlorate	*Gambusia holbrooki*
Crane et al. (2005)	Ammonium perchlorate	*Pimphales promelas*
Crane et al. (2006)	Methimazole	*P. promelas*
Elsalini and Rohr (2003)	Methimazole, Potassium perchlorate, Phenylthiourea, Propylthiouracil	*Danio rerio*
Li et al. (2009b)	Amitrole	*Gobiocypris rarus*
Li et al. (2011)	Magnesium perchlorate	*Gobiocypris rarus*
Liu et al. (2006)	Sodium arsenate, Sodium perchlorate	*D. rerio*
Mukhi and Patiño (2007)	Sodium perchlorate	*D. rerio*
Mukhi et al. (2005)	Ammonium perchlorate	*D. rerio*
Mukhi et al. (2007)	Perchlorate	*D. rerio*
Park et al. (2006)	Sodium perchlorate	*Gambusia holbrooki*
Patino et al. (2003)	Ammonium perchlorate	*D. rerio*
Raldúa and Babin (2009)	Multiple: methimazole, propylthiouracil, potassium perchlorate	*D. rerio*
Schmidt et al. (2012)	Potassium perchlorate	*D. rerio*
Thienpont et al. (2011)	Multiple	*D. rerio*
van der Ven et al. (2006)	Propylthiouracil	*D. rerio*
Manzon and Youson (1997)	Potassium perchlorate	*Petromyzon marinus*

Table D.2: Amphibian studies evaluated for occurrence of NMDR in thyroid.

Reference	Chemical	Species
Carlsson and Norrgren (2007)	PTU	*Xenopus tropicalis*
Coady et al. (2010)	Methimazole, T4	*X. laevis*
Degitz et al. (2005)	PTU, methimazole, T4	*X. laevis*
Goleman et al. (2002a)	Ammonium perchlorate	*X. laevis*
Goleman et al. (2002b)	Ammonium perchlorate	*X. laevis*
Goleman and Carr (2006)	Ammonium perchlorate, sodium perchlorate, ammonium chloride	*X. laevis*
Hornung et al. (2010)	PTU, methimazole, perchlorate	*X. laevis* (thyroid glands)
Hu et al. (2006)	perchlorate	*X. laevis*
Oka et al. (2009)	T4, PTU	*Rana rugosa*
Opitz et al. (2005)	T4, PTU, ETU	*X. laevis*
Opitz et al. (2006a)	ETU	*X. laevis*
Tietge et al. (2005)	Sodium perchlorate	*X. laevis*
Tietge et al. (2013)	Mercaptobenzothiazole	*X. laevis*
Tindall et al. (2007)	Methimazole	*X. tropicalis*

FISH

FISH Sodium-μIodine Symporter (NIS) Inhibitor Study Synopses

Liu et al. (2006) evaluated the effects of 10 and 100 mg/L sodium perchlorate on juvenile male zebrafish. The exposure duration was 90 days, with thyroid samples taken at 10, 30, 60, and 90 days for histological analysis. Statistically significant changes were observed in percent colloid area, epithelial cell height, follicular angiogenesis, and hyperplasia, and were all monotonically related to perchlorate concentration.

Mukhi et al. (2007) exposed larval zebrafish (3 days post-fertilization) for 30 days to 100 and 250 mg/L sodium perchlorate (as perchlorate) and evaluated thyroid histology and gonadal histology and sex ratios (not covered in this analysis). Epithelial cell height and colloid depletion were significantly increased in monotonic fashion.

Mukhi et al. (2005) exposed 12 week old zebrafish for 12 weeks to 10, 100, 1,000, and 10,000 µg/L ammonium perchlorate (as perchlorate). Fish were sampled at 2, 4, 8, and 12 weeks for thyroid histology analysis, at 2 and 12 weeks for immunohistochemical staining of the colloidal T4 ring, and at 12 weeks for whole body T4 concentrations. Follicular cell height and follicular angiogenesis increased monotonically with perchlorate concentrations and with duration of exposure. The colloidal T4 ring staining was statistically significantly reduced monotonically with perchlorate concentrations and with duration of exposure. There were no statistically significant changes in whole body T4 concentrations.

Elsalini and Rohr (2003) employed an immunohistochemical staining procedure to evaluate the presence of T4 in histological sections of thyroid follicles in zebrafish exposed to four model goitrogens, including perchlorate, methimazole, propylthiourea, and propylthiouracil. The zebrafish embryos were exposed beginning at 6 hours post fertilization and sampled 3, 4, or 5 days later. Qualitative analysis suggests that the reductions in T4 occurred according to the exposure concentration, although no quantification was attempted. **Raldúa and Babin (2009)** used a similar, but quantitative approach with immunofluorescent detection in zebrafish whole mounts. This study exposed 2 day old zebrafish for 3 days to seven concentrations of methimazole, propylthiouracil, and potassium perchlorate. All three of these chemicals statistically significantly reduced T4 detection monotonically. Thienpont et al. (2011) further applied the immunofluorescent method to numerous chemicals, including methimazole and perchlorate, which replicated the monotonic responses observed in the previous study.

The effects of perchlorate on thyroid histology were generally monotonic. One study that reported a potential NMDR, **Patino et al. (2003)**, showed increased follicular cell hyperplasia and colloid depletion at 18 ppm as compared to the controls, whereas 677 ppm had no effect. This apparent NMDR is caveated by the facts that there was general toxicity (reported loss of appetite; lethargy) and acute lethality observed in the 677 ppm treatment. Furthermore, the 677 ppm histopathology samples were taken 4 weeks earlier than the 18 ppm samples preventing time-matched comparisons to controls and other treatments. Thus, this apparent NMDR is highly uncertain and likely related to toxicity of the high concentration.

Of the three zebrafish studies that included TH measurements (**Schmidt et al., 2012**; **Mukhi and Patiño, 2007**; **Mukhi et al., 2005**), none of the studies reported non-monotonic effects.

Crane et al. (2005) evaluated the effects of ammonium perchlorate on fathead minnows and reported an apparent NMDR with follicular cell height where exposure to 1 and 10 ppm were significantly larger than the controls, but exposure to 100 ppm resulted in statistically significantly reduced follicular cell heights compared to the 10 ppm exposure. However, this study also observed statistically significant reductions in growth at 10 and 100 ppm of about 40 and 60 % respectively, indicating that the two higher exposure concentrations were likely at toxic concentrations.

Park et al. (2006) evaluated the effects of 1, 10, and 100 ppm perchlorate (as sodium perchlorate) on Eastern mosquitofish and found that, after 42 days of exposure, follicular cell hypertrophy, follicular cell hyperplasia, and colloid depletion all increased monotonically with perchlorate concentration. In a similarly conducted study, **Bradford et al. (2005)** exposed mosquitofish to 0.1, 1.0, 10, 100, and 1,000 ppm perchlorate and evaluated thyroid histology and whole body T4 concentrations following 2, 10, and 30 days of exposure. Similar to **Park et al. (2006)** follicular cell height and hyperplasia, follicle hypertrophy, and colloid depletion all were affected in a monotonic fashion. The magnitude and prevalence of the effects both increase with duration of exposure. Whole body T4 measurements were largely inconclusive and highly variable. The authors note that whole body T4 measurements are subject to methodological and sampling problems and suggest that circulating T4 would be a better measurement.

Li et al. (2011) evaluated the effects of 5 and 50 ppb perchlorate on the expression of genes for the iodothyronine deiodinase enzymes (D1, D2, and D3) and sodium iodide symporter (NIS) in the brain and liver of adult Chinese rare minnows. They also measured D2 and NIS in larvae. Expression of these genes was analyzed following 7, 14, and 21 days of exposure. Circulating T4 and T3 were measured in adult plasma at the end of the 21 day exposure. D2 and NIS expression in the larvae were monotonic within each sampling event, although the direction of change was inconsistent among the different sampling times.

Effects on hepatic expression of D1, D2, D3, and NIS were monotonic in males within each sampling event, although direction of change was again inconsistent among the sampling times. For example, NIS expression at 7 days was monotonically reduced to about 25% of control expression, while NIS expression at 21 days was monotonically increased to over 200% of control expression. Changes in D1 and D2 expression in females were monotonic. However, D3 and NIS expression in female livers were monotonic at 7 and 21 days, but non-monotonic at 14 days.

Effects on brain expression of D2, D3, and NIS were monotonic in females, with D3 showing strong and consistent reductions at most time points. The same three genes in males exhibited both monotonic and NMDR of varying magnitude and direction, depending upon the sample time.

Circulating T4 was unchanged in both sexes at both concentrations, and T3 was reduced at the 50 ppb concentration in males only.

The complicated response patterns, both monotonic and non-monotonic, observed in this study makes the data difficult to analyze.

Manzon and Youson (1997) exposed larval and metamorphing sea lampreys of variable sizes to 0.01 and 0.05 % potassium perchlorate and measured the effects on circulating T3 and T4. Serum T3 was reduced monotonically in all developmental stages and sizes, while T4 was reduced monotonically in larval and metamorphing lamprey of the two smaller size classes. No NMDRs were observed.

Fish TPO Inhibitor Study Synopses

van der Ven et al. (2006) exposed zebrafish adults and offspring to 1, 10, and 100 mg/L PTU in a partial life cycle study. Exposure to PTU resulted in concentration dependent changes in thyroid-specific endpoints in the adults, including: thyroid follicle hypertrophy and hyperplasia, follicular cell hypertrophy, colloid depletion, and reduced circulating T4 and T3. Similar histological observations were made in the F1 generation as well as reduced scale thickness, an indicator of inhibition of thyroid hormone-dependent metamorphosis. No NMDRs were observed.

Schmidt and Braunbeck (2011) evaluated the effects of PTU on zebrafish using a 5 week exposure of larvae to 2.5, 10, 25, and 50 mg/L. Histological effects on thyroid follicles and follicular cells occurred in a concentration dependent manner, as did the reduction in whole body T4 concentrations. Morphometric analysis of the pituitary revealed an NMDR in total pituitary area and adenohypohyseal area, which was highest in the 25 mg/L treatment. However, the normalized area of the adenohyphysis, using the ratio of the adeno- to the neuro-hypophysis, increased in a monotonic manner.

Crane et al. (2006) evaluated the effects of 32, 100, and 320 µg/L methimazole (MMI) in an 84 day study starting with less than 24 hour old fathead minnow embryos. Thyroid-specific endpoints were evaluated at 28, 56, and 84 days. Whole body T4 and T3 measurements were made at 28 and 56 days, while circulating concentrations were measured at 84 days. An NMDR was observed in the 28 day measurements of whole body T4 concentrations where T4 was significantly reduced at 32 and 100 ug/L, but not at 320 µg/L. At 56 days, whole body T4 was increased at 320 µg/L only. Whole body T3 was reduced at 320 µg/L at 28 days, but a slight decrease was observed only at the 100 µg/L at 56 days. Circulating thyroid hormone levels at 84 days were not statistically analyzed in the females due to sample size problems, but there was no significant differences observed in the T4 and T3 measurements in the males.

The authors suggest that the NMDRs in whole body thyroid hormone measurements could be due to either compensatory mechanisms or from altered thyroid hormone metabolism. No data are presented in support of either suggestion.

Li et al. (2009b) evaluated the effects of 1 to 10,000 ng/L amitrole using juvenile Chinese rare minnows exposed for 28 days. Circulating T4 and T3 were unaffected in both sexes at all treatment concentrations. Hepatic expression of TTR and D1 were monotonically upregulated in males at almost all concentrations, but only TTR was upregulated in females and only at the highest concentration. D2 was upregulated in both sexes at all concentrations with the exception of the highest concentration exposure to females. While female expression of D2 was flat across the remaining exposure concentrations, D2 expression in males was elevated higher in the 1, 10, and 100 ng/L concentrations than in the 1,000 and 10,000 ng/L concentrations, suggestive of an NMDR. However, histological analysis revealed cellular degeneration in the liver in both sexes exposed to 10,000 ng/L.

Expression of D2 in the brain of males increased monotonically, but there was no effect in the females. TR-alpha expression in the brain of males was reduced in all but the lowest concentration, while there was no effect on female expression.

AMPHIBIAN

Amphibian NIS Inhibitor Study Synopses

Goleman et al. (2002a) evaluated the effects of a 70 day exposure of 59 µg/L and 14 mg/L perchlorate (administered as ammonium perchlorate) on *X. laevis* embryos. Metamorphic development was significantly inhibited and whole body T4 concentrations were significantly reduced monotonically. Perchlorate contamination of the control exposure solutions (0.02 µg/L) and unusually slow metamorphic development of controls confounds this study.

Goleman et al. (2002b) evaluated the effects of ammonium perchlorate using *X. laevis*. Embryos were exposed for 70 days to nine concentrations ranging from 1.18 µg/L to 1,175 mg/L AP. Forelimb emergence and complete tail resorption, indicators of metamorphic progression, were both reduced monotonically.

Goleman and Carr (2006) evaluated the effects of 2 concentrations sodium perchlorate and ammonium perchlorate (38 µg/L and 14.04 mg/L) on *X. laevis* embryos exposed for 70 days. Metamorphic development and thyroid gland histology were the primary endpoints. Both chemicals monotonically reduced hindlimb length and developmental stage indicating inhibition of metamorphosis. Further, exposure to both chemicals resulted in monotonic increases in colloid depletion and follicular cell hypertrophy and hyperplasia.

Hu et al. (2006) evaluated the effects of sodium perchlorate at 4 concentrations ranging from 1 µg/L to 1 mg/L as perchlorate on *X. laevis* larvae using a 69 day exposure. Conventional histology of the thyroid gland, immunocytochemistry of colloidal T4 ring, and development were evaluated. Forelimb emergence, tail resorption, and hindlimb length were all reduced monotonically. Follicular cell height and colloid depletion increased monotonically, while colloidal T4 ring staining intensity was reduced monotonically.

Tietge et al. (2005) conducted two experiments which exposed *X. laevis* to sodium perchlorate. In the first experiment, 2 different developmental stages were exposed to 16, 63, 250, 1,000, and 4,000 µg/L perchlorate (as sodium perchlorate) for 8 and 14 days. Metamorphic development and thyroid gland histology were the primary endpoints, both of which were affected monotonically regardless of developmental stage. In the second experiment, a single developmental stage was exposed to 8, 16, 32, 63, and 125 µg/L perchlorate throughout metamorphic development with a subsample taken at 14 days to evaluate thyroid gland size. Thyroid gland size increased monotonically with perchlorate concentrations, and time to complete metamorphosis was statistically significantly longer in the highest test concentration, indicating developmental delay.

Amphibian TPO Inhibitor Study Synopses

Degitz et al. (2005) evaluated the effects of methimazole, propylthiouracil, and T4 on thyroid histology and metamorphic development in *X. laevis* with two different tests. The first experiment evaluated the effects of 8 and 14 day exposures of 5 concentrations of methimazole (6.25, 12.5, 25, 50, and 100 mg/L) and 5 concentrations of PTU (1.25, 2.5, 5.0, 10, and 20 mg/L) on two different developmental stages. Both metamorphic development and thyroid histology were statistically significantly affected monotonically with exposure concentrations, regardless of the developmental stage tested. The second experiment exposed two different developmental stages for 14 and 21 days to PTU at the same concentrations as above and to T4 (0.25, 0.50, 1.0, 2.0 and 4.0 µg/L). PTU statistically significantly retarded development monotonically, while T4 significantly accelerated development monotonically.

Opitz et al. (2005) reports the result of 6 laboratory ring test of a specific *X. laevis* protocol that evaluates the effect of the test chemical for 28 days on metamorphic development. Ten experiments were conducted exposing *X laevis* larvae to 5.0, 10, 25, 50, and 100 mg/L ethylenethiourea (ETU). All ten studies observed inhibition of metamorphosis in a concentration dependent manner.

Opitz et al. (2006a) evaluated the developmental, histological, and molecular effects of ETU exposure on *X. laevis* larvae. This study exposed the animals to 1.0, 2.5, 10, 25, and 50 mg/L for up to 90 days. Metamorphic development, as determined by developmental stage and forelimb emergence, was retarded monotonically. Most of the morphological and histological endpoints changed monotonically with the exception of follicular cell height, which was statistically significantly decreased at 10 mg/L, but increased at 25 and 50 mg/L. This apparent NMDR is in contrast with the other histological data and is not interpreted further in the discussion. Since the 50 mg/L treatment arrested development, changes in gene expression were excluded from further consideration. With this qualification, expression of pitutitary TSHβ and TSHα genes were increased at 25 mg/L. Expression of brain TRβ was not statistically significantly changed by any treatment.

Coady et al. (2010) evaluated the effects of 4.0, 16.5, and 50 mg/L methimazole and 0.1, 0.6, and 3.0 µg/L T4 on *X. laevis* for 21 days according the Amphibian Metamorphosis Assay protocol (**OECD, 2009**; **U.S. EPA, 2009**). Methimazole statistically significantly reduced snout-vent length, hindlimb length, and developmental stage and altered thyroid gland histology monotonically. T4 significantly accelerated metamorphic development and increased hindlimb length, while inducing histological changes typical of hyperthyroidism (thyroid gland atrophy, reduced follicular size and colloid).

Carlsson and Norrgren (2007) evaluated the effects of 2, 5, 10, 20, and 75 mg/L 6-PTU on *X. tropicalis* larvae in a 14 day exposure. Metamorphic development was delayed monotonically. Diffuse hypertrophy of the thyroid gland and follicular cell height increased monotonically. Follicular lumen area increased through 10 mg/L but began to decrease at 20 mg/L. This apparent NMDR is attributable to collapse of the follicle, an observation made in other studies. Reinforcing this explanation is the fact that the follicular lumen areas at the 75 mg/L PTU concentration were excluded from analysis due to severe follicular lumen collapse, which prevented accurate measurements.

Tietge et al. (2013) evaluated the effects of a novel TPO inhibitor, 2-mercaptobenzothiazole (MBT) using 7 and 21 day assays with *X. laevis* larvae. Five concentrations were used in each study and ranged from 18 to 357ug/L and 23 to 435 µg/L in the 7 and 21 day studies, respectively. Both studies evaluated thyroid gland histology and metamorphic development. The 7 day study also evaluated circulating TSH and T4, iodinated compounds in the thyroid gland, and expression of the sodium iodide symporter (NIS) gene in the thyroid gland. Metamorphic development was inhibited monotonically in the 21 day study and several measures of thyroid gland histology were affected monotonically in both studies. In the 7 day study, circulating T4 was reduced, and circulating TSH was increased monotonically. NIS gene expression in the thyroid gland increased monotonically, while thyroidal monoiodotyrosine, diiodotyrosine, T3, and T4 were all reduced monotonically.

Tindall et al. (2007) evaluated the effects of methimazole on iodine uptake in *X. tropicalis* embryos using 1, 5, and 10 mM concentrations. Iodine uptake increased monotonically over the concentration range tested.

Oka et al. (2009) evaluated the effects of PTU on *Rana rugosa* larvae using a 28 day exposure. This study used 19, 75, and 150 mg/L PTU and evaluated metamorphic development and thyroid gland histology. Development was retarded monotonically. Histological effects appear to have responded monotonically, based on a qualitative assessment.

Hornung et al. (2010) describe a novel method using cultured thyroid glands of *X. laevis* to evaluate the effects of a test chemical on T4 release to the culture media. This method uses a fixed bTSH concentration to provide static stimulation of the gland to produce and release T4. This approach was used to evaluate several concentrations of methimazole (0.1-10 mg/L), 6-propylthiouracil (0.15–15 mg/L), and perchlorate (0.005–5 mg/l). All three chemicals inhibited T4 release monotonically.

Table D.3: Fish studies not evaluated for occurrence of NMDR in thyroid.

Reference	Chemical	Species	Reason for not including in analysis
Bernhardt and von Hippel (2008)	Sodium perchlorate	*Gastersteus aculeatus*	no thyroid endpoints
Bernhardt et al. (2006)	Sodium perchlorate	*Gastersteus aculeatus*	no thyroid endpoints
Chan and Chan (2012)	BDE-47, TBBPA, BPA	*Danio rerio*	chemical with wrong moa
Chen et al. (2012)	BDE-209	*D. rerio*	chemical with wrong moa
Coimbra et al. (2005)	Endosulfan, Aroclor 1254	*Oreochromis niloticus*	chemical with wrong moa
(Ebbesson et al., 1998)	Propylthiouracil	*Oncorhychus kisutch*	single concentration
Lema et al. (2008)	PBDE-47	*Pimephales promelas*	chemical with wrong moa
Li et al. (2009a)	Acetochlor	*Gobiocypris rarus*	chemical with wrong moa
Liu et al. (2008)	Perchlorate	*D. rerio*	single concentration
Liu et al. (2011)	Prochloraz, Propylthiouracil	*D. rerio*	single concentration
Manzon and Youson (1997)	Potassium perchlorate	*Petromyzon marinus*	single concentration
Morgado et al. (2009)	DES, Ioxynil, PTU	*Parus aurata*	single concentration; dietary exposure
Pelayo et al. (2012)	BPA	*D. rerio*	chemical with wrong moa
Shi et al. (2009)	PFOS	*D. rerio*	chemical with wrong moa
Teles et al. (2005)	β-naphthoflavone	*Anguilla anguilla*	chemical with wrong moa
Wang et al. (2013)	Tris phosphate (TDCPP)	*D. rerio*	chemical with wrong moa
Yan et al. (2012)	Mycosystin	*D. rerio*	chemical with wrong moa
Yu et al. (2010)	DE-71	*D. rerio*	chemical with wrong moa
Zaccaroni et al. (2009)	Nonylphenol	*Carassius auratus*	chemical with wrong moa
Zhang et al. (2009)	Tributyltin	*Sebastiscus marmoratus*	chemical with wrong moa
Zheng et al. (2012)	BDE-47 and 2 analogs: 6-OH-BDE-47 6-MeO-BDE-47	*D. rerio*	chemical with wrong moa

Table D.4: Amphibian studies not evaluated for occurrence of NMDR in thyroid.

Reference	Chemical	Species	Reason for not including in analysis
Fini et al. (2007)	Methimazole, perchlorate, IOP	*Xenopus laevis*	single concentration
Fort et al. (2000)	Multiple chemicals	*X. laevis*	Binary mixtures; single concentrations
Helbing et al. (2007a)	T3, T4, PTU, methimazole, perchlorate	*X. laevis*	single concentration
Helbing et al. (2007b)	T3, T4, PTU, methimazole, perchlorate	*X. laevis*	single concentration
Miranda et al. (1995)	KClO4	*Bufo arenarum*	single concentration
Mitsui et al. (2006)	PTU	*X. tropicalis*	single concentration
Opitz et al. (2006b)	Perchlorate; ETU	*X. laevis*	single concentration
Opitz et al. (2009)	Perchlorate, ETU	*X. laevis*	single concentration
(Opitz and Kloas, 2010)	T4, perchlorate	*X. laevis*	single concentration
(Ortiz-Santaliestra and Sparling, 2007)	Nitrate + perchlorate	*Rana sphenocephala*	high mortality
Saka et al. (2012)	T4, PTU	*X. tropicalis*	single concentration
Serrano et al. (2010)	PTU, methimazole, perchlorate	*X. laevis*	single concentration
Sparling and Harvey (2006)	Ammonium bicarbonate, ammonium perchlorate	*R. pipiens*	no thyroid specific endpoints
Theodorakis et al. (2006)	Perchlorate	*Campostoma anomalum, Acris crepitans*	field study
Tietge et al. (2010)	PTU, methimazole, perchlorate	*X. laevis*	single concentration
Zhang et al. (2006)	T4, T3, PTU, perchlorate, methimazole	*X.laevis*	single concentration

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