

5.4 AQUATIC ANIMALS

5.4.1 Fish

5.4.1.1 *Perchlorate Residues in Native Fish*

5.4.1.1.1 Introduction

In order to confirm perchlorate exposure, fish were collected from aquatic environments within the study area and analyzed for the presence of the perchlorate anion. The goals of this effort were to assess the exposure (and potential effects) of perchlorate in fish as well as to assess the exposure potential for perchlorate in humans through the consumption of contaminated fish.

5.4.1.1.2 Methodology

5.4.1.1.2.1 Preliminary Assessments

The first assessment of perchlorate residues in fish was conducted in May, 2001. At that time, fish were collected from various streams within the Lake Waco and Lake Belton Watersheds. Based on the results of the May, 2001 sampling event, an immediate follow-up sampling was requested in August/September, 2001. This fall sampling was on fish fillets and also included sediment collections.

5.4.1.1.2.2 Routine Monitoring

At each location where fish were captured, at least two water samples were collected in precleaned glass vials (Wheaton) from just under the water surface. Water samples were then analyzed for perchlorate according to **Appendix X**. In the streams, fish were sampled with a backpack shocking apparatus set at a current of 2-4 amps and a frequency of 30-60 cps, or were collected by seining. All fish collected from the streams were identified to species, frozen whole in liquid nitrogen on site, and kept at -20° C for perchlorate analysis. The fish were then dried under a fume hood, ground in a Waring blender, and extracted on an Accelerated Solvent Extraction apparatus, using water as the solvent. Extracts were then analyzed for perchlorate according to **Appendix X**. The sampling sites included Station Creek (T20-T23), South Bosque River at Highway 317 (T16), S Creek at Highway 317 (T15), and a tributary of Harris Creek off US Highway 84 (T17) just west of McGregor, TX. These sites are shown on **Figure 5-143**. In Lake Waco and Lake Belton, fish were collected with an aluminum boat equipped with an electroshocking apparatus. All boat electroshocking was carried out within 50 m of the shoreline. Fish were collected from two to four stations per lake, with each station comprising an approximately 100-150 m stretch of shoreline. At least three water samples (as above) were collected at each station where fish were collected. Fish collected from the lakes were identified to species, anesthetized on ice, and sacrificed by cervical scission. Fillets were then collected, frozen in liquid nitrogen on site, and kept at -20 °C until perchlorate analysis according to **Appendix X**. In some cases, heads were also collected because laboratory data (see Section 5.4.1.3) indicated that perchlorate may

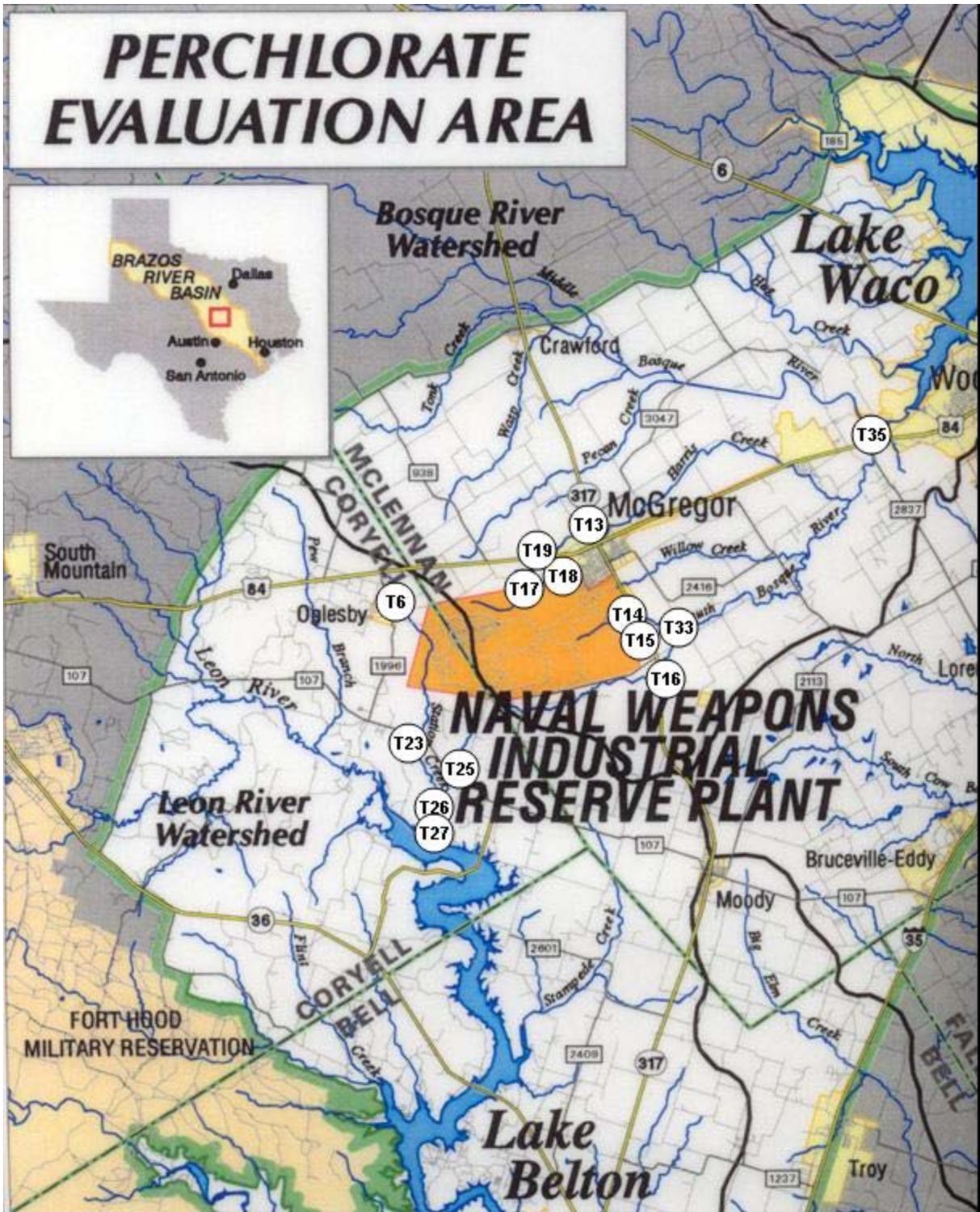


Figure 5-143
Map of Study Area Illustrating the Approximate Locations where Fish, Water, and Sediment Pore Water Samples Were Collected

accumulate in the head at concentrations that are greater than in the fillet. Because perchlorate concentrations in the fillets may have been near the detection limit, heads were analyzed because it was hypothesized that the concentrations in the head may be a more sensitive indicator than perchlorate accumulation in the edible portions of the fish.

5.4.1.1.3 Data

5.4.1.1.3.1 Preliminary Assessments

Results of the initial assessment of perchlorate residues in fish are presented in **Table 5-27**. Because of the location of thyroid follicles in fish and the size of the fish captured, this initial assessment focused on residues analyses of fish heads.

**Table 5-27
Preliminary Data on Perchlorate Concentrations in Fish Heads within the Lake
Waco and Lake Belton Watershed**

Location	Species	Perchlorate (ng/g)
South Bosque at Indian Trail (T33)	Suckers (4)	2030 [930]
		1940 [890]
		2110 [970]
		2290 [1050]
	Catfish (3)	1360 [630]
		1010 [470]
		1850 [850]
Largemouth Bass (1)	600 [280]	
Harris Creek at Highway 317 (T13)	Sunfish (4)	740 [340]
		890 [410]
		2-ND
	Catfish (4)	730 [350]
		190 [90]
		290 [140]
		270 [130]
	Largemouth Bass (1)	ND
Harris Creek at Highway 84 (T19)	Sunfish (4)	710 [340]
		360 [170]
		1060 [500]
		690 [330]
Station Creek at Highway 107 (T23)	Sunfish (4)	ND
		540 [240]
		290 [130]
		2730 [1230]

ND = not detected by the analytical procedure [detection limit in dry tissue = 100 ppb]
 Perchlorate concentrations are in ng/g (ppb) expressed based on tissue dry weight. Data represent the results of analyses of individual tissues (heads) with the number of analyses indicated parenthetically. Respective wet weight concentrations are presented in brackets. Samples were collected in May, 2001 from areas previously identified through water quality analyses as having received perchlorate.

As part of the preliminary assessment, filets from catchable-size fish were also collected within the Leon and South Bosque Rivers and their tributaries during August/September, 2001. At the time of fish collection, water samples (**Table 5-28**) and sediment pore water samples (**Table 5-29**) were also collected. All samples (59 fillet samples from 6 locations, 18 sediment samples from 7 locations, and 41 water samples from 16 locations) were analyzed for perchlorate contamination using ion chromatography.

Only four (4) filets tested positive for quantifiable perchlorate residues (**Table 5-30**). Two additional fish had trace quantities of perchlorate (perchlorate levels detectable, but below the limit of quantitation). Perchlorate was not detected in sediment samples from locations where fish were collected or from additional areas within the Lake Waco and Lake Belton Watersheds. Perchlorate was detected in several water samples from locations within the two Watersheds, consistent with our previous data indicating various levels of perchlorate contamination in flowing water.

Table 5-28
Perchlorate Concentrations in Surface Water within the Lake Waco and Lake
Belton Watersheds Corresponding to the Dates of Fish Sampling during a
Preliminary Assessment

Location	Sample ID	Perchlorate (ng/mL)	Collection Date
Harris Creek at Highway 317 (T13)	00490	ND	08/28/01
	00253	ND	09/06/01*
	00247	ND	09/06/01*
Harris Creek at Oglesby Road (T17)	00457	ND	08/28/01
	00264	ND	09/06/01*
	00265	ND	09/06/01*
Harris Creek at Highway 84 (T19)	00483	ND	08/28/01
Spring on Oglesby Road (T18)	00462	62	08/28/01
	00263	50	09/06/01
	00261	48	09/06/01
Unnamed Tributary near WWTP at Highway 317 (T14)	00453	ND	08/28/01
	00271	36	09/06/01
	00270	34	09/06/01
S Creek at Highway 317 (T15)	02303	388	09/07/01
	02245	402	09/07/01
South Bosque at Highway 317 (T16)	00452	ND	08/28/01
	02301	ND	09/06/01*
	02302	ND	09/06/01*
South Bosque at Indian Trail (T33)	00469	36	08/29/01*
	00477	34	08/29/01*
South Bosque at Highway 84 (T35)	00492	ND	08/28/01*
	00459	ND	08/28/01*
	00520	ND	08/28/01*
	00466	ND	08/28/01*
	00481	ND	08/28/01*
Station Creek at Highway 107 (T23)	00451	ND	08/28/01
	02277	32	09/06/01
	02276	29	09/06/01
	02314	30	09/07/01
	02319	32	09/07/01
Station Creek at Leon River (T27)	00267	ND	09/06/01
	00266	ND	09/06/01
	02298	ND	09/07/01*
	02294	ND	09/07/01*
Station Creek at Old River Road (T26)	00464	ND	08/29/01
	00275	ND	09/06/01
	00246	ND	09/06/01
Station Creek at Oglesby Road (T6)	00455	ND	08/28/01
Onion Creek at Highway 107 (T25)	00450	33	08/28/01

*Date of fish collection

ND = not detected (detection limit = 1 ppb)

Table 5-29
Perchlorate Concentrations in Sediment Pore Water within the Lake Waco and
Lake Belton Watersheds Corresponding to the Dates of Fish Sampling during a
Preliminary Assessment

Location	Sample ID	Perchlorate (ng/mL)	Collection Date
Harris Creek at Highway 317 (T13)	00234	ND	09/06/01*
	00235	ND	09/06/01*
Harris Creek at Oglesby Road (T17)	02244	ND	09/06/01
Unnamed Tributary near WWTP at Highway 317 (T14)	02304	ND	09/06/01
	02321	ND	09/06/01
S Creek at Highway 317 (T15)	02300	ND	09/07/01
	02320	ND	09/07/01
South Bosque at Indian Trail (T33)	00478	ND	08/29/01*
	00479	ND	08/29/01*
South Bosque at Highway 84 (T35)	00468	ND	08/28/01*
	00448	ND	08/28/01*
	00519	ND	08/28/01*
	00420	ND	08/28/01*
	00518	ND	08/28/01*
Station Creek at Leon River (T27)	00269	ND	09/06/01
	00268	ND	09/06/01
	02243	ND	09/07/01*
	02272	ND	09/07/01*

*Date of fish collection.

ND = not detected (detection limit = 4 ppb)

Table 5-30
Perchlorate Concentrations in Fish Fillets Within the Lake Waco and Lake Belton Watersheds

Location	Species	Perchlorate (ng/g)
Harris Creek at Highway 317 (T13)	Green sunfish (2)	2-ND
	Largemouth Bass (4)	1-TRACE 3-ND
	Yellow bullhead (1)	1-ND
Harris Creek at Oglesby Road (T17)	Green sunfish (2)	2-ND
	Yellow bullhead (1)	1-TRACE
South Bosque at Highway 84 (T35)	Carp (2)	2-ND
	Green sunfish (1)	1-ND
	Sucker (1)	1-ND
	Channel catfish (6)	6-ND
	Large mouth bass (6)	1-690 5-ND
	White bass (1)	1-ND
	Flat head catfish (1)	1-ND
	Sunfish (2)	2-ND
South Bosque at Indian Trail (T33)	Largemouth bass (16)	1-590 15-ND
	Sunfish (6)	6-ND
	Sucker (1)	1-ND
South Bosque at Highway 317 (T16)	Green sunfish (2)	1-260 1-ND
	Channel catfish (4)	1-260 3-ND
Station Creek at Leon River (T27)	Channel catfish (4)	1-260 3-ND
	Yellow bullhead (1)	1-ND

ND = not detected by the analytical procedure [detection limit in wet tissue = 170 ppb].
 TRACE = perchlorate was detected, but the perchlorate concentration in the extract was below the limit of quantitation (2.5 ppb).

(Perchlorate concentrations are in ng/g (ppb) expressed based on tissue wet weight. Data represent the results of analyses of individual tissues (fillets), except where indicated, with the number of analyses indicated parenthetically. Samples were collected in August-September, 2001 from areas previously identified through water quality analyses as having received perchlorate.)

5.4.1.1.3.2 Routine Monitoring

Water and tissue concentrations for perchlorate are presented for samples collected in October, 2001 (**Table 5-31** and **Table 5-32**, respectively), March, 2002 (**Table 5-33**), and August, 2002 (**Table 5-34** and **Table 5-35**). Sites sampled are shown in **Figure 5-144**. Perchlorate water concentrations for March are indicated in **Appendix C**. All body burden data are expressed on a per dry weight basis, and were based on whole body analysis. Most of the fish collected from streams in the study area were not of legal size (>8 inches).

Table 5-31
Average (\pm Standard Deviation) Perchlorate Concentration in Water Samples
Collected at Various Streams Near NWIRP in October, 2001

Sampling Site	Conc. (ppb)
Coryell Creek (T36)	ND
Wasp Creek (T2)	ND
Harris Creek at Highway 84 (T19)	28.8 \pm 4.4
Station Creek at Highway 107 (T23)	23.6 \pm 0.9
S Creek at Highway 317 (T15)	202.6 \pm 23.5
South Bosque at Highway 317 (T16)	10.8 \pm 9.3

n = 3

ND = not detected (method detection limit = 1ppb)

Table 5-32
Whole Body Concentrations of Perchlorate (ppb) in Fish Collected Near NWIRP in
October, 2001

Species	Collection Sites				
	Coryell Creek (T36)	Wasp Creek (T2)	Harris Creek (T19)	South Bosque (T16)	Station Creek (T23)
Green sunfish	ND (n=2)	ND (n=3)	170 \pm 159 (n=5)	81 \pm 181 (n=5)	ND (n=2)
Longear sunfish	ND (n=4)	ND (n=2)	-	88 \pm 95 (n=8)	ND (n=1)
Stoneroller minnow	ND (n=1)	ND (n=3)	-	-	79 \pm 136 (n=7)
Yellow bullhead	ND (n=7)	-	-	ND (n=1)	ND (n=3)
Redfin shiner	-	-	-	-	ND (n=1)
Blackspotted topminnow	-	-	-	ND (n=2)	-
Blacktail shiner	-	ND (n=1)	-	ND (n=1)	220 (n=1)
Black bullhead	-	-	ND (n=1)	-	ND (n=2)
Mosquitofish	-	ND (n=1)	-	ND (n=1) 1198 (n=1)	-
Largemouth bass	-	-	-	-	ND (n=2)

ND = not detected

- = fish not collected

Table 5-33
Whole Body Concentrations of Perchlorate (ppb) in Fish Collected Near NWIRP in
March, 2002

Species	Collection Sites			
	Coryell Creek (T36)	Harris Creek (T19)	South Bosque (T16)	Station Creek (T23)
Green sunfish	ND (n=6)	ND (n=9)	ND (n=8)	ND (n=11)
Longear sunfish	ND (n=5)	-	ND (n=11)	ND (n=5)
Stoneroller fish	ND (n=12)	-	ND (n=6)	ND (n=2)
Yellow bullhead	ND (n=6)	-	ND (n=2)	ND (n=4) 250, 125
Redfin shiner	ND (n=2)	-	-	ND (n=8)
Blacktail shiner	ND (n=2)	-	ND (n=5)	-
Black bullhead	-	ND (n=4)	-	-
Darter	-	-	ND (n=1)	-
Mosquitofish	-	-	ND (n=1)	-
Largemouth bass	-	-	ND (n=1) 373, 246	-
Bluegill sunfish	-	-	-	ND (n=1)

ND = not detected
 - = fish not collected

Table 5-34
Average (\pm Standard Deviation) Perchlorate Concentration in Water Samples
Collected at Various Streams Near NWIRP in August, 2002

Site	Conc. (ppb)
Coryell Creek (T36)	ND
Wasp Creek (T2)	ND
Harris Creek at Highway 84 (T19)	24.2 \pm 0.1
Station Creek at Highway 107 (T23)	14.2 \pm 0.9
S Creek at Highway 317 (T15)	343.1 \pm 0.1
South Bosque at Highway 317 (T16)	ND

ND = not detected (method detection limit = 1ppb)
 n = 3

Table 5-35
Whole Body Concentrations of Perchlorate (ppb) in Fish Collected Near NWIRP in August, 2002

Species	Collection Sites			
	Coryell Creek (T36)	Wasp Creek (T2)	South Bosque (T16)	Station Creek (T23)
Green sunfish	ND (n=5)	569 ± 205 (n=3) ND (n=2)	ND (n=5)	ND (n=4) 541 (n=1)
Stoneroller minnows	ND (n=5)	ND (n=3) 1593 ± 335 (n=2)	ND (n=4) 3153 (n=1)	ND (n=5)

ND = not detected

In general, detection of perchlorate in fish tissues was sporadic, but, when it was found, tissue concentrations were higher than that found in the water.

Results from analysis of edible portions (fillets) of fish collected from Lakes Belton and Waco are presented in **Table 5-36** (February, 2002) and **Table 5-37** (March, 2002). Perchlorate was not detected in any water samples. All fish were of legal size (>8 inches for most fish), although some of the species collected (drum, spotted gar) are not typically consumed by humans. Refer to **Plate 6** for sampling locations in Lake Belton and Lake Waco.

Generally, the trends in the lakes are similar to that in the streams: sporadic detection of perchlorate at concentrations in the fillets greater than in the water. For black crappie, perchlorate was more likely to be detected in the head than in the fillet. Perchlorate was more likely to be detected in the fillets than in the heads for largemouth bass, spotted gar, and drum.

Table 5-36
Summary of Perchlorate Analysis for Fish Collected from Lakes Waco and Belton
in February, 2002

Lake	Location	Species	Number Collected ^a	Number Detects ^b	Concentration ^c
Lake Waco	Site 1 ^d	Largemouth Bass	10	1	990
		Carp	3	0	
		Channel Catfish	4	0	
		White Bass	3	0	
		Drum	2	0	
		Smallmouth Buffalo	2	0	
		Shad	4	0	
		White Crappie	1	0	
		Bluegill	3	0	
		Warmouth Sunfish	2	0	
	Site 2 ^d	Largemouth Bass	1	1	550
		Channel Catfish	1	0	
		River Carpsucker	1	0	
Lake Belton	Site 1 ^d	Largemouth Bass	4	0	
		Black Crappie	3	0	
		Shad	1	0	
		Smallmouth Buffalo	1	0	
		Spotted Gar	1	0	
	Site 2 ^d	Largemouth Bass	2	1	720
		Channel Catfish	5	1	970
		White Bass	3	0	
		Black Crappie	1	0	
		Spotted Gar	1	0	
Drum	2	0			

^aTotal number of fish collected at that site

^bNumber of fish in which perchlorate was above the detection limit (330 ppb)

^cConcentration (ppb) of perchlorate in fish for which perchlorate was above the detection limit

^dGPS coordinates for the sampling sites are as follows: Lake Waco Site 1 – N31°30.752' W 097° 15.130'; Lake Waco Site 2 – N 31° 31.702' W 097° 14.739'; Lake Belton Site 1 - N31° 16.090' W 097° 28.789'; Lake Belton Site 2 - N31° 15.986 W 097° 28.484'.

Table 5-37
Summary of Perchlorate Analysis for Fish Fillets and Heads Collected from Lakes
Waco and Belton in May, 2002

Location ^a	Species	# detected / # analyzed		Concentration ^b (ppb)	
		fillet	head	fillet	head
Lake Waco					
Site 1	White bass	0/3	0/3		
	Largemouth bass	0/1	1/1		4560
	Drum	0/3	0/3		
	Flathead catfish	0/1	0/1		
	Channel catfish	0/3	1/3		626
	Black crappie	0/3	1/3		1960
	Bluegill sunfish	0/1	0/1		
Site 2	Carp	0/2	-		
	Largemouth bass	1/3	0/3	1400	
	Drum	0/3	0/1		
	Shad	0/1	0/1		
	Redear sunfish	0/1	0/1		
	Black crappie	0/3	2/3		1770; 1510
Lake Belton					
Site 1	Spotted gar	2/2	0/1	2040; 3960	
	Carp	0/2	-		
	Smallmouth buffalo	0/1	-		
	Largemouth bass	0/2	0/1		
	White bass	0/2	0/2		
	Drum	0/3	0/2		
	Shad	0/3	0/3		
	Bluegill sunfish	0/2	0/2		
	Flathead catfish	0/1	-		
Site 2	Spotted gar	1/3	-	1910	
	Drum	1/3	0/3	2740	
	White crappie	0/1	0/1		
	Shad	0/3	0/3		
	Channel catfish	0/2	-		

^aSites are the same as described in **Table 5-36**

^bConcentration (ppb) of perchlorate in fish for which perchlorate was above the detection limit.
 Perchlorate concentrations are in ng/g (ppb) tissue dry weight

5.4.1.1.4 Discussion

The results presented here are similar to those found in Smith et al. (2001), who also sporadically detected perchlorate in fish tissues at concentrations that were greater than in the water. Possible explanations for this may be that a) perchlorate uptake occurs via other routes than from the water (e.g., via algae, see Section 5.3.2), or b) there is a high temporal variation in perchlorate levels in the water over time, and elimination from fish tissues may lag behind decreases in ambient water concentrations (illustrated in laboratory studies presented in this report). In general, the frequency of detection of perchlorate in fish tissues seems to be associated with proximity to the NWIRP. It is important to note that most (> 95%) of the fish caught in streams in the study area were not of legal size (> 8 inches), thus they would not likely be consumed by humans.

Curiously, perchlorate was found in 3 of 5 green sunfish and 2 of 5 stonerollers collected from Wasp Creek in August, 2002. There is no logical explanation for this occurrence. Based on findings during this study, including groundwater and surface water interaction, there is no known migration pathway for perchlorate from NWIRP to result in exposure of fish in this stream. Wasp Creek does not originate on NWIRP grounds, as do Station and Harris Creeks and the South Bosque River. Wasp Creek is located in a separate sub-watershed, the boundary of which is approximately 1.5 miles from NWIRP, and the distance from known groundwater plumes of perchlorate is substantial. Nonetheless, the analytical quality assurance for these fish samples was satisfactory, so there was no analytical reason to qualify the data. Therefore, these data create a source of uncertainty for the potential exposure of fish to perchlorate in Wasp Creek.

In the lakes, perchlorate was occasionally detected in the fillets of several sport fish, most notably largemouth bass and channel catfish. However, because the U.S. EPA has not yet instituted an acceptable consumption limit or benchmark dose for food-borne exposure to perchlorate, it is not possible to determine if this is within acceptable limits. In addition, for black crappie, perchlorate was detected in the head but not the fillet on several instances. Although the head is not usually considered an edible tissue, the lack of perchlorate detection in the fillets of this species may be due to detection limits rather than absence of perchlorate in edible tissues.

Both laboratory and field data indicate that human exposure to perchlorate through consumption of fish caught in the study watersheds is possible, but highly unlikely. This conclusion is based on (1) where perchlorate occurs in surface water and the size of fish supported by those streams, (2) the preferential accumulation of perchlorate in rarely consumed tissue (head) rather than the fillet, (3) fillet residue data on larger fish caught near the exposure points and the lakes, and (4) laboratory studies on perchlorate tissue distributions in exposed fish.

5.4.1.2 Thyroid Histology in Native Fish

5.4.1.2.1 Introduction

Perchlorate is known to affect thyroid function, causing subsequent hormone disruption and potential perturbations of metabolic activities. In teleost fish, thyroid hormones promote growth (Donaldson et al., 1979), stimulate early gonadal development (Cyr and Eales, 1996), stimulate steroidogenesis (Sullivan et al., 1989; Cyr and Eales, 1996), and initiate metamorphosis in many fish (Inui and Miwa, 1985; Inui et al., 1995). Because of the involvement of thyroid hormone in fitness components such as reproduction, growth, and development, thyroid endocrine disruption could ultimately be manifested at the population, community or ecosystem level of biological organization. In most vertebrates, thyroid hormone also interacts with retinoid signaling pathways (retinoids are a class compounds that include vitamin A and derivative, and regulate limb development in embryonic and larval vertebrates [Chatterjee and Tata, 1992]).

Thus, the focus of this research is to explore spatial and temporal patterns of perchlorate concentrations in water and fish, as well as examining effects of perchlorate on thyroid function in fish.

5.4.1.2.2 Methodology

Central stonerollers (*Campostoma anomalum*) and green sunfish (*Lepomis cyanellus*) were collected from the streams listed in Section 5.4.1.1, as well as from Wasp Creek at Highway 317 (just south of Crawford, TX) and Coryell Creek (chosen as reference sites). Fish were collected with a backpack electroshocking device, as described above. They were anesthetized on site, and the heads were preserved in Bouin's fixative for histological analysis. Fish heads were left in Bouin's fixative for 2 days to decalcify and fix tissues. After 2 days, the Bouin's was removed and cassette storage containers were placed under gently running water overnight to continuously rinse the tissue. Storage containers were then filled with 70% ethanol, and the ethanol was replaced daily until the yellow color in the tissues disappeared. Tissues were processed with the Tissue-Tek V.I.P. 2000 Processor (Miles Laboratories, Elkhart, IN) and then embedded in paraffin. Sections were cut with a microtome (5 um) and mounted on microscope slides. The slides were then stained with hematoxylin and Eosin Y for observation following Humason (1967).

Slides were observed with a light microscope and the number of follicles per section and the height of the follicular epithelium were measured in 10 sections randomly selected from each fish in each exposure group. In each fish, a total of 10 follicles were selected, and the height of 10 epithelial cells per follicle were measured. Five follicles from the rostral end (the first section to have at least 5 follicles) and from the caudal end (the section with at least 5 follicles not included in the first group) of the thyroid follicular region were measured. Individual sections were randomly chosen based on the overall quality (whole follicles, no folds or tears) of the section. For each follicle, epithelial cell height was measured using a micrometer. For each fish, the percent hyperplastic follicles, and percent of follicles with depleted colloid, were also noted.

5.4.1.2.3 Data

The results from histological analysis of thyroid tissues for fish collected from NWIRP streams in October 2001 and August 2002 are presented in **Figure 5-145** and **Figure 5-146**, respectively. The hypothesis of differences among sites was tested using a nonparametric (Kruskal-Wallis) test, because the data did not meet the criteria for using parametric statistics (normality and homogeneity of variances). The data from the October 2001 sampling indicate that, for thyroid follicle epithelial heights, the level of effect is in the order of Harris Creek > Wasp Creek > Station Creek > Coryell Creek.

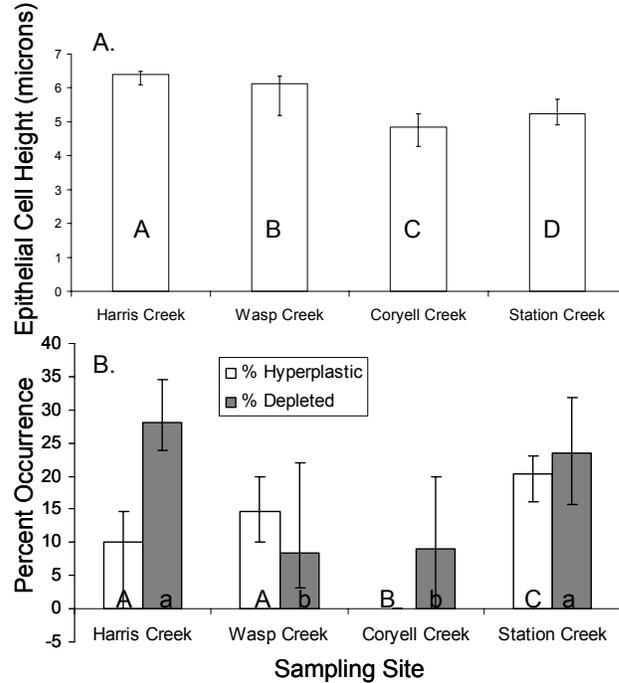


Figure 5-145
Thyroid Follicular Epithelial Cell Heights (A) and Percent Occurrence of Follicles with Hyperplastic Epithelia or Depleted Colloid (B) from Stoneroller Minnows Collected from Various NWIRP Streams in October, 2001

Bars and error bars represent medians with first and third quartiles. Bars labeled with different letters are statically significantly different ($P < 0.05$, Kruskal-Wallis test).

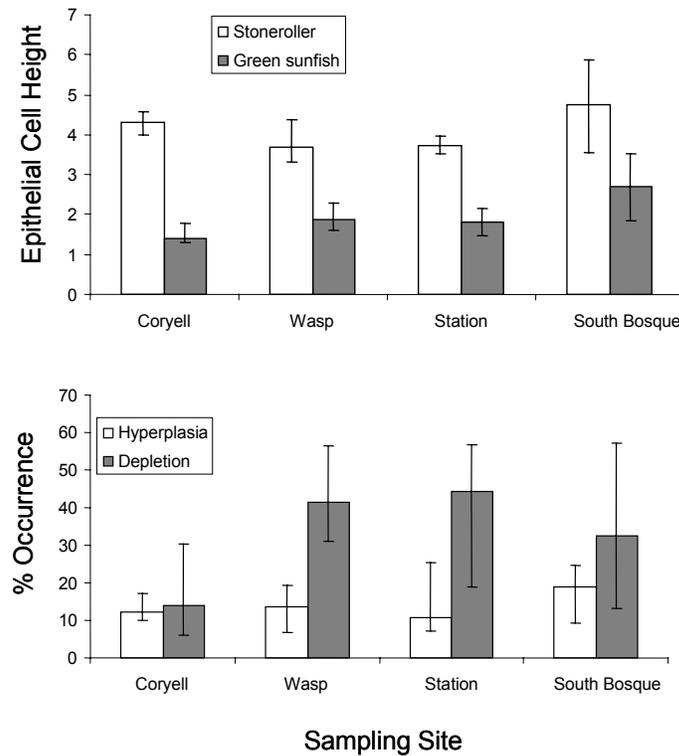


Figure 5-146
Thyroid Follicular Epithelial Cell Heights for Stonerollers and Green Sunfish (A)
and Percent Occurrence of Follicles with Hyperplastic Epithelia or Depleted Colloid
in Stonerollers (B) Collected from Various NWIRP Streams in August, 2002

Bars and error bars represent medians with first and third quartiles. Bars labeled with different letters are statically significantly different ($P < 0.05$, Kruskal-Wallis test).

For percent occurrence of hyperplastic follicles, the relative levels of effect were Station Creek > Wasp Creek = Harris Creek > Coryell Creek, and for percent occurrence of follicles with depleted colloid the levels of effect were Harris Creek = Station Creek > Wasp Creek = Coryell Creek. In addition, all three metrics were used to classify the sites as to magnitude of effect by calculating an average rank for each individual metric (**Table 5-38**). For example, for the follicle epithelial cell heights, Harris Creek was given a rank of “1”, Wasp Creek was ranked a “2”, Station Creek a “3” and Coryell Creek a “4”. In the case of ties (no statistically significant differences) the tied sites were given average ranks. For instance, for depleted colloid, Harris Creek and Station Creek were each given a rank of 1.5 (average of 1 and 2) and Wasp Creek and Coryell Creek were each given a rank of 3.5 (average of 3 and 4). For each site, the ranks were averaged over all three metrics (epithelial cell height, percent hyperplastic follicles, percent depleted colloid) in order to determine relative overall level of impact. According to this method, the order of overall level of impact was Harris Creek > Station Creek > Wasp Creek > Coryell Creek (**Table 5-38**).

Table 5-38
Ranking of NWIRP Sites According to Magnitude of Response for Several Thyroid
Histopathological Metrics

Sampling Site	Rank				Average Rank
	Epithelial height		Hyperplastic follicles	Depleted colloid	
	Stoneroller	Green sunfish			
A. October 2001					
Station Creek (T23)	3	-	1	1.5	1.8
Harris Creek (T19)	1	-	2.5	1.5	2.3
Wasp Creek (T2)	2	-	2.5	3.5	2.7
Coryell Creek (T36)	4	-	4	3.5	3.8
B. August 2002					
Station Creek (T23)	3.5	2.5	2	2.5	2.625
South Bosque (T16)	1.5	1	2	2.5	1.75
Wasp Creek (T2)	3.5	2.5	2	2.5	2.625
Coryell Creek (T36)	1.5	3	4	2.5	2.75

* See **Figure 5-144** for sampling locations.

For the data from the August 2002 sampling, the epithelial cell heights were calculated for green sunfish and stonerollers, while the percent occurrence for hyperplasia and colloid depletion were calculated for stonerollers alone. The data for stoneroller and green sunfish epithelial cell heights indicate relative levels of effect in the order of Coryell Creek = South Bosque > Wasp Creek = Station Creek, and South Bosque > Station Creek = Wasp Creek > Coryell Creek, respectively. For percent depleted colloid, the relative levels of effect was in the order of Wasp Creek = Station Creek = South Bosque > Coryell Creek (**Table 5-38**). There were no statistically significant differences between sites for percent hyperplastic follicles, although Station Creek and South Bosque had the highest values (**Figure 5-146**). The average ranks among sites (averaged over all metrics) were in the order of South Bosque > Station Creek = Wasp Creek > Coryell Creek.

5.4.1.2.4 Discussion

Overall, the level of effect was associated with proximity to the NWIRP. Fish collected from streams that originated on or passed through the NWIRP grounds, especially Harris Creek and the South Bosque River, showed more thyroid histology impacts than fish from other areas.

Although both Wasp Creek and Coryell Creek were intended to be reference sites, results for fish collected from Wasp Creek were not entirely consistent with fish collected from Coryell Creek. The Wasp Creek fish seemed to be intermediate between the NWIRP streams and Coryell Creek. Although perchlorate and thyroid histology impacts were found in some fish collected from Wasp Creek (discussed in Section 5.4.1.1), there was no logical explanation for the perchlorate residue data. Additionally, thyroid histological impacts were identified in some fish from Wasp Creek that had no detectable concentrations of perchlorate in tissue.

In comparing the epithelial cell heights between green sunfish and stonerollers, the patterns seen in the sunfish more closely match the perchlorate contamination histories of the streams. Wasp Creek does not originate on NWIRP grounds, as do Station and Harris Creeks and the South Bosque River. Wasp Creek is located in a separate sub-watershed from NWIRP, and the distance from known groundwater plumes of perchlorate is substantial. There is no known migration pathway for perchlorate from NWIRP to result in exposure of fish in this stream. Therefore, data indicating thyroid histopathological impacts in fish from Wasp Creek create a source of uncertainty, as some other component in the water (or the lack of some nutrient) could be impacting thyroid histology.

In conclusion, the thyroid histopathology data presented suggest that these fish were impacted by perchlorate, although perchlorate is not the only environmental contaminant that could influence changes in thyroid histology (a lack of iodine, for example, could also influence thyroid structure and function). The conclusion that fish were impacted by perchlorate is supported by an integrated approach using multiple vitiaes (“weight of evidence” Suter, 1993). This approach is more robust than looking at just one biomarker of effect. Although thyroid histology is adversely impacted in fish from streams near NWIRP, laboratory studies indicate that fish can recover from these effects as perchlorate is removed. In addition, it is not clear whether the negative thyroid impacts observed are sufficient to cause changes in fish populations in the study streams. In general, fish were abundant in most of the streams sampled, suggesting that any impact of perchlorate was not being manifested at the community or population level.

5.4.1.3 Kinetics of Perchlorate Uptake and Depuration in Laboratory Fish

5.4.1.3.1 Introduction

Although the mechanisms by which perchlorate acts on the thyroid have been well established, there is little information on the dose-response of perchlorate in ecologically relevant species. Uptake and depuration kinetics of perchlorate may provide a better understanding of perchlorate fate and effects in fishes, as well as providing information on the dose-response of perchlorate in environment. Also, there is little information on tissue distribution of perchlorate in fish. Such information would be useful in determining which tissue to use for biomonitoring purposes, and would be vital for developing physiologically-based toxicokinetic models in fish. The rates of perchlorate uptake and depuration in channel catfish (*Ictalurus punctatus*) and eastern mosquitofish (*Gambusia holbrooki*) were examined following short-term exposure. Because perchlorate release is often characterized by the short-term release of relatively high concentrations of toxicant

(Smith et al., 2001), and because depuration of highly water-soluble compounds is expected to occur relatively rapidly, the experimental design employed a limited-time exposure and high perchlorate concentration.

5.4.1.3.2 Methodology

5.4.1.3.2.1 Uptake

Female eastern mosquitofish (*Gambusia affinis*) and channel catfish (*Ictalurus punctatus*) were exposed to 0, 0.1, 1, 10, 100, and 1000 mg/L sodium perchlorate for 2, 10, and 30 days with 5 replicate aquaria of each exposure group. After these exposure times, perchlorate was determined in the whole bodies. Channel catfish were exposed to 100 mg/L sodium perchlorate for 5 days with 20 replicates to determine the uptake of perchlorate into specific tissues. Sex was not determined in the catfish. Mosquitofish and catfish were obtained from commercial hatcheries and treated with antibiotics prior to exposure. All fish were allowed to acclimate to the laboratory conditions for at least 5 days prior to exposure.

Fifteen mosquitofish were randomly assigned to each 15 L aquaria water consisting of 60 mg/L Instant Ocean sea salts in deionized water with the appropriate amount of sodium perchlorate stock solution added to each test aquarium. Fish were fed commercial fish flake food daily. Water samples were also taken on the first day and last day of each exposure and once a week for the longer exposures and analyzed to determine actual perchlorate concentration in each aquarium. Following each exposure, the mosquitofish were euthanized in MS-222 (3-aminobenzoic acid ethyl ester and NaHCO₃, each mixed in distilled water at 1.5 g/L) and the length and weight of each fish was recorded. Approximately 8 fish from each aquaria were frozen in liquid nitrogen for perchlorate analysis, with the remainder used for other analyses.

One catfish was assigned to each 15 L aquarium. Aquaria water was composed of reconstituted fresh water as above with the appropriate amount of sodium perchlorate stock solution added to each test aquarium. Fish were fed commercial shrimp pellet food each day, and feeding time, daily observations of behavior, fish health and condition, and number of dead fish were recorded. Water changes and water quality analyses were as discussed above. Following exposure, the catfish were euthanized in 1.5 g/L MS-222 and The kidney (KY), liver (LV), gill (GL), gonad (GD), gastrointestinal tract (GI), head (HD), and fillet (FL) were dissected out and preserved in liquid nitrogen for determination of perchlorate concentrations into each tissue.

For perchlorate analysis, fish were desiccated under a fume hood, ground in a Warring blender, and extracted in an Accelerated Solvent Extractor, using water as the solvent. Extracts were cleaned using silica solid phase extraction (SPE) cartridges. The diluted extracts were analyzed as described in **Appendix X**.

5.4.1.3.2.2 Elimination

This experiment was carried out in a static renewal design system. In this system, fish were exposed to 100 mg/L sodium perchlorate solution for 2 days, then transferred to

pre-cleaned aquaria containing clean water. Fifty-two mosquitofish and 20 catfish were initially exposed to 100 mg/L sodium perchlorate for 2 days, and then transferred to aquaria containing clean water. At 0, 1, 2, 5, and 10 days of elimination, 4 catfish and 5 mosquitofish were removed, rinsed in 3 successive tanks filled with DI water to remove sodium perchlorate on their skin and gills, and anesthetized with MS-222 (1.5 g/L). Anesthetized fish were dissected quickly to collect tissues (muscle, liver, gastrointestinal (GI) tract (stomach + intestine), gills and head). All tissue samples were immediately frozen in liquid nitrogen, and stored at -70 °C until perchlorate analysis. In a subsequent experiment, catfish were again exposed for 2 days and allowed to depurate for 20 days. At 0 and 20 days, we dissected 2-day exposed catfish following above-mentioned procedure. For mosquitofish, we followed above methods, except that we used whole body to determine the concentration of perchlorate. After exposing mosquitofish to 100 mg/L sodium perchlorate for 2 days, they were transferred to clean water. Seven fish in each replicate were collected at 0, 1, 2, 5, 10, and 20 days. All samples collected from catfish and mosquitofish were also rinsed with DI water 3 times to remove potential perchlorate on surface of samples, weighed, air dried and extracted with distilled, deionized water and analyzed using ion chromatography, as described in **Appendix X**. Because the size of liver was too small to extract perchlorate from samples, we combined two catfish livers.

For perchlorate analysis, fish were thawed and desiccated under a fume hood, ground in a Waring blender. They were then extracted in an Accelerated Solvent Extractor, using water as the solvent. Extracts were analyzed for perchlorate as described in **Appendix X**.

5.4.1.3.2.3 Rate Constant Determination

The uptake rate constants (K_1) were calculated from $K_1 = K_2 \cdot C_m / C_w$; C_m is the concentration in tissues and C_w is the concentration in water. When the fish are transferred to clean water, the elimination rate constant (K_2) was determined using a non-linear regression model: $C_{mT} = C_{mo} e^{-K_2 T}$ where C_{mo} is the concentration of perchlorate in the selected tissue at the beginning of the experiment, C_{mT} the concentration of perchlorate in the selected tissue at the end of the experiment, and T the time of elimination (day). The half-life ($T_{1/2}$) of perchlorate in tissues was estimated according to the equation $T_{1/2} = -\ln 0.5 / K_2$.

5.4.1.3.3 Data

5.4.1.3.3.1 Uptake of Perchlorate in Mosquitofish

Perchlorate was not detected in mosquitofish exposed to the lowest concentrations of perchlorate (0, 0.1, and 1 mg/L sodium perchlorate), regardless of the exposure time. Perchlorate was detected in mosquitofish exposed to 10, 100, and 1000 mg/L sodium perchlorate, but the whole body tissue concentrations were approximately 10 times less than the exposure concentration (**Figure 5-147**).

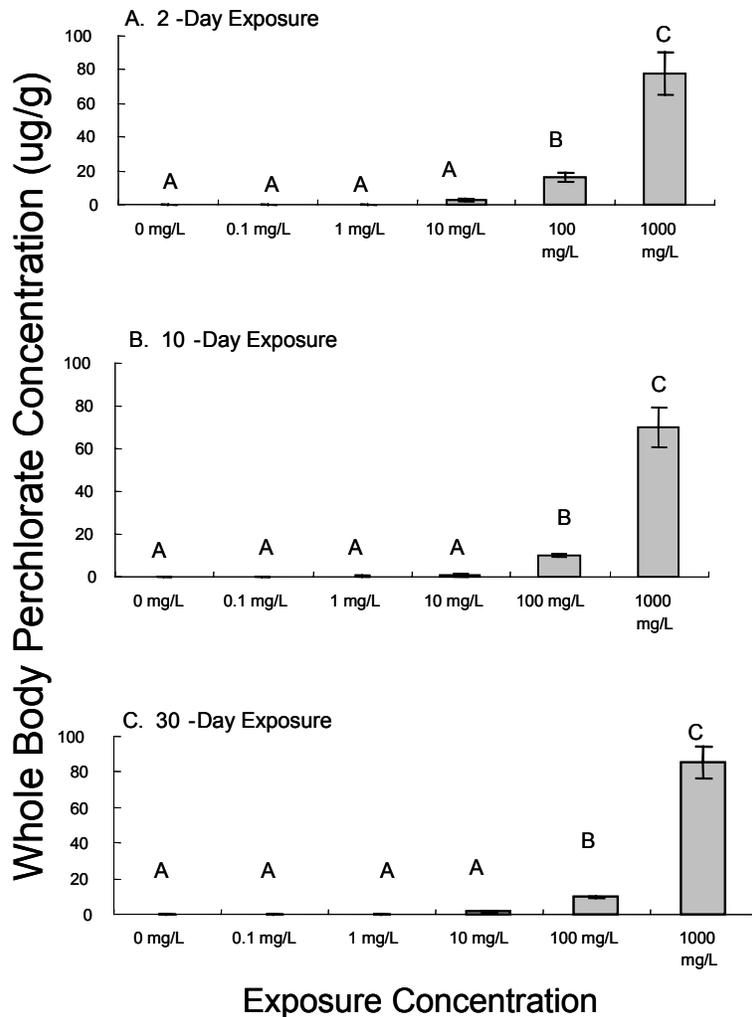


Figure 5-147
Perchlorate Uptake in Mosquitofish Exposed to Various Concentrations of Sodium Perchlorate for 2 days (A), 10 days (B), and 30 days (C)

Perchlorate concentrations are based upon the wet weight of the sample. Bars labeled with different letters are significantly different ($P < 0.05$, Kruskal-Wallis test). (Bars are the mean \pm standard error, $n = 5$).

Bioconcentration factors for the uptake of perchlorate into whole mosquitofish were determined. Mosquitofish had very low BCF across all exposure concentrations. The highest BCF were in fish exposed to 10 and 100 mg/L sodium perchlorate with BCF of 0.15 and 0.12, respectively. BCF for all other exposure concentrations were less than 0.01.

Because the data for the uptake of perchlorate into mosquitofish were non-normally distributed and no transformations could be found that would normalize the data or homogenize the variances, differences between the concentrations in whole body mosquitofish were analyzed by the Kruskal-Wallis test. There was no difference in the uptake of perchlorate depending upon the exposure time ($P = 1.000$), which leads to the assumption that perchlorate does not bioconcentrate in fish. There was, however, a

difference in perchlorate uptake depending upon the concentration of the exposure dose ($P < 0.001$).

Comparisons of the treatments with the control group within each time period indicated there were differences in uptake in fish exposed to the 2 highest concentrations of perchlorate. Fish exposed to 100 mg/L sodium perchlorate in all 3 time periods had a significantly higher uptake than the control group ($P = 0.05$). Fish exposed to 1000 mg/L also had significantly higher uptake compared to the control group ($P = 0.01$).

Comparisons of treatment medians within each time period showed that mosquitofish exposed to 1000 mg/L sodium perchlorate in all 3 treatment time periods had a significantly greater uptake of perchlorate into the tissues ($P = 0.05$) compared to mosquitofish exposed to 0, 0.1, 1 mg/L sodium perchlorate. Fish exposed to 100 mg/L sodium perchlorate also had a significantly higher uptake of perchlorate into the tissues after the 2 day and 10 day exposures ($P = 0.10$) as well as after 30 days of exposure ($P = 0.05$) compared to fish exposed to the lower concentrations.

5.4.1.3.3.2 Uptake of Perchlorate in Catfish

In catfish, the largest concentrations of perchlorate were found in the head and in the fillet, while perchlorate was not detected in the gonad tissue. The gastrointestinal tract, liver, kidney, and gill all took up less perchlorate relative to the head and fillet. Based upon the results of this first experiment, the exposure was repeated with 5 catfish in order to look at sections of the head and the muscle and the skin separately in order to determine if there is a specific region in which perchlorate accumulates. **Figure 5-148** shows the uptake of perchlorate in catfish.

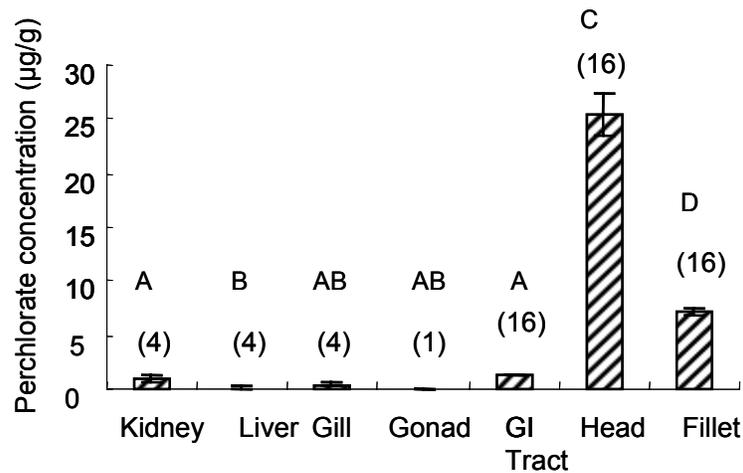


Figure 5-148
Uptake of Perchlorate in Tissues of Catfish Exposed to 100 mg/L Sodium Perchlorate for 5 days

Sample sizes are given in parentheses above each bar. (Bars are the mean of the actual concentrations \pm standard error). Sample sizes < 16 indicate that tissues were pooled for that individual tissue to provide enough material for analysis. Bars labeled with the same letter are not statistically significantly different ($P > 0.05$, Kruskal-Wallis test).

The BCF's for all tissue types indicate that bioconcentration was very low. The highest BCF was in the head of the catfish with a BCF of 0.21, and the 3 head sections had BCF's of about 0.14, indicating that the differences in water concentration may have attributed to the differences in the head concentration compared to the head sections. The next highest BCF was in the fillet with a BCF of 0.060, and the skin and muscle tissues had a BCF of 0.14 and 0.03, respectively. All other BCF's were less than 0.01 (kidney, liver, gill, gonad, and gastrointestinal tract).

Because of the large variances in the uptake of perchlorate in catfish tissues, data were logged transformed and then analyzed by a 1-way ANOVA. Results from the 1-way ANOVA on the logged transformed data indicated there was a difference in perchlorate uptake into the various catfish tissues, depending upon which tissues were examined ($P < 0.001$). A comparison of the treatment means showed that most tissues had significantly different uptakes relative to all other tissues examined.

5.4.1.3.3.3 Perchlorate Elimination Rate in Catfish

The measured concentration of perchlorate, $83,843 \pm 4,165$ ppb in aquaria during exposure periods was close to the nominal concentration of sodium perchlorate, 100 ppm. In depuration period, the measured concentrations of perchlorate eliminated by the fish into the aquarium water were 69.7 ± 82.8 ppb, 121 ± 110 ppb, 138 ± 115 ppb, 107 ± 91 ppb, 71.2 ± 59.3 ppb, 56.0 ± 44.7 ppb, and 40.5 ± 29.5 ppb at 0, 1, 2, 3, 5, 7, and 10 days. The concentration of perchlorate in aquarium water during the first two days of the depuration period increased. No water change was performed during this period. Thereafter, the perchlorate concentration in aquarium water decreased due to the rapid elimination of perchlorate from body and the water changes, which occurred every other day. Accumulated perchlorate concentrations in each tissue during 2-day exposure period were showed at 4.1%, 21.1%, 13.3%, 14.2%, and 21.3% of exposure concentration in fillet, liver, GI tract, gills, and head, respectively (**Figure 5-149**).

The uptake rate constants (K_1) for 2 day exposure calculated in this study showed highest value in head and liver among the tissues, and fillet showed the lowest value (see **Table 5-39**).

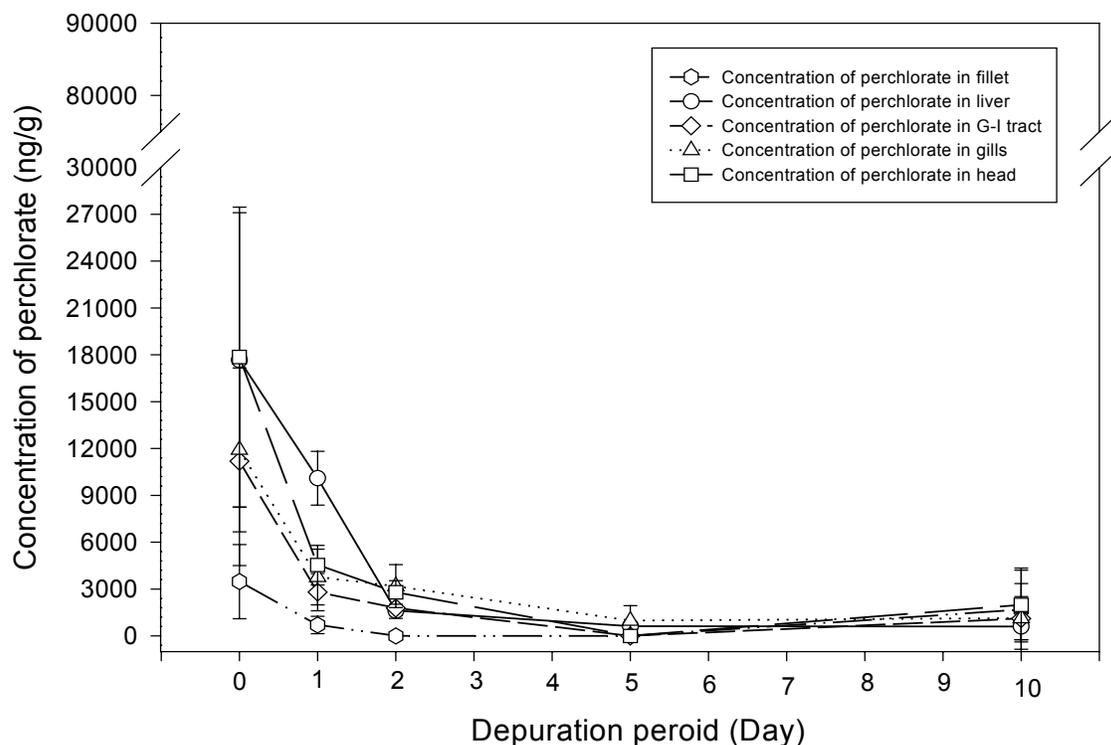


Figure 5-149
Perchlorate Contents ($\mu\text{g/g}$, Wet Weight) in each Tissue of Catfish (*Ictalurus punctatus*) during Recovery Period (10 Days) after Exposure to 100 g/L Sodium Perchlorate for 2 Days in a Static Design System (n=4, except Liver (n=2))

Table 5-39
Perchlorate Toxicokinetic Indexes in Tissues of the Channel Catfish (*Ictalurus punctatus*) and Whole Body of Mosquitofish (*Gambusia holbrooki*)

Tissues	Uptake Rate Constant K_1 (day^{-1})	Elimination Rate Constant K_2 (day^{-1})	Half-life $T_{1/2}$ (day)
Catfish Fillet	0.07	1.67	0.41
Liver	0.17	0.79	0.88
G-I tract	0.16	1.22	0.57
Gills	0.13	0.88	0.79
Head	0.26	1.22	0.57
Mosquitofish	0.10	0.76	0.91

After one day of elimination, more than 50% of perchlorate was eliminated from the fillet without skin (80%), head (75%), G-I tract (74%), gills (68%), and liver (43%) (**Figure 5-149**). However, perchlorate was still present in all tissues in at least some individuals

after 10 days. The uptake (K_1), elimination rate (K_2) and the half-life ($T_{1/2}$) of perchlorate were calculated and are presented in table 1. The elimination rate constant was most rapid from fillet and slowest from liver tissue. The elimination of perchlorate residues from fillet was approximately two times faster than liver. That would be related to a low half-life in fillet (0.41 day). In all tissues of catfish, perchlorate concentrations significantly varied with time (significance levels were $P < 0.05$), except in liver ($P = 0.14$). Observed concentrations of ClO_4^- in tissues of catfish decreased with time (**Figure 5-149**).

Because the perchlorate was not totally eliminated after 10 days, we expanded the depuration period to 20 days. The measured concentration of perchlorate during 2-day exposure periods was $78,274 \pm 6,199$ ppb, which is also close to nominal concentration of sodium perchlorate (100ppm). In depuration period, the measured concentrations of perchlorate eliminated into the water were 1.5 ± 3.4 ppb, 70.2 ± 28.4 ppb, 89.3 ± 33.8 ppb, 11.3 ± 5.0 ppb, 6.1 ± 3.6 ppb, 0.8 ± 1.8 , and 0 ± 0 ppb, at 0, 1, 2, 6, 10, 14, and 18 days. At day 20, perchlorate was not detected at fillet, gill, and head; however, there were still detections of perchlorate in liver and G-I tract of catfish at 2 and 0.6 mg/L, respectively.

5.4.1.3.3.4 Perchlorate Elimination Rate in Mosquitofish

Analysis of perchlorate in aquarium water during a 2 day exposure period showed that the actual concentration of perchlorate was $73,720 \pm 2,005$ ppb and was close to the nominal concentration of sodium perchlorate (100 ppm). In depuration period, the measured concentrations of perchlorate eliminated into the water were 0 ± 0 ppb, 12.4 ± 0.9 ppb, 16.2 ± 1.3 ppb, 9.8 ± 0.8 ppb, 0 ± 0 ppb, 0 ± 0 ppb, 0 ± 0 ppb, and 0 ± 0 ppb at 0, 1, 2, 5, 10, 14, 18, and 20 days, respectively. The concentration of perchlorate in the water also increased during the first two days of the depuration, indicating that perchlorate was depurated from the body into water. No water change occurred during this period. The kinetics of perchlorate elimination in mosquitofish is presented in **Figure 5-150**.

Perchlorate concentrations in fish at the end of the 2-day exposure period were $9,880 \pm 1,440$. The accumulation of perchlorate after 2-day exposure was showed at 13.4%. The uptake (K_1), elimination rate (K_2) and the half-life ($T_{1/2}$) of perchlorate were calculated and are presented in **Table 5-39**. Perchlorate concentrations in fish also varied significantly with time ($P < 0.05$) and observed concentration of ClO_4^- in mosquitofish also decreased with time (**Figure 5-150**).

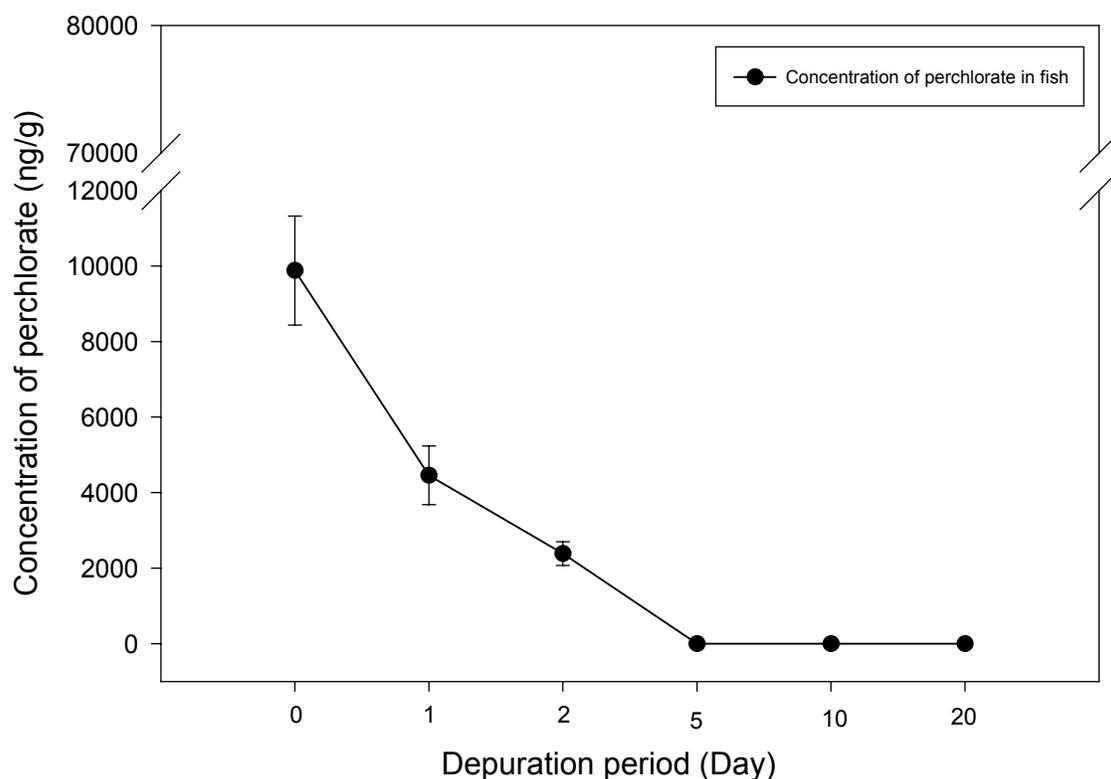


Figure 5-150
Perchlorate Contents ($\mu\text{g/g}$, Wet Weight) in Mosquitofish (*Gambusia holbrooki*) during Recovery Period (20 Days) after Exposure to 100 g/L Sodium Perchlorate for 2 Days in a Static Design System (n=5)

5.4.1.3.4 Discussion

In general, these results indicate that perchlorate is taken up and reaches steady state relatively rapidly, in accordance with one of the initial hypotheses. However, perchlorate was detected in fish only at elevated exposure concentrations and was not detected in fish that were exposed to environmentally relevant concentrations of perchlorate. Fish that were exposed to perchlorate in the laboratory did not bioaccumulate perchlorate; the tissue concentration of perchlorate was much lower than the exposure concentration of perchlorate. Contrary to this study, perchlorate concentrations in wild fish may be greater than those in the water.

Results from the catfish exposure to perchlorate were consistent with field studies. Under laboratory conditions, the highest perchlorate concentrations were found in the head, with the fillet having the next highest concentration. Perchlorate concentrations in the head were more than 3 times higher than the perchlorate concentrations in the fillets, and other tissues had very small amounts of perchlorate in comparison to the concentrations found in the head. Field studies were conducted to determine the amount of perchlorate present in the heads and fillets of several species of fish. These studies also found that the head

had much higher concentrations of perchlorate than did the fillets, at least for black crappie (Section 5.4.1.1).

The results of this study indicate that the head and fillet are the most important tissues to analyze when determining the uptake of perchlorate into fish. Although perchlorate did accumulate in other tissues in the catfish (kidney, liver, gill, and gastrointestinal tract), these tissues had very low concentrations of perchlorate compared to the head and the fillet. This may have important implications for dietary exposure of perchlorate to humans eating contaminated fish, because the muscle tissue usually constitutes the majority of tissue consumed. This information is necessary for determining ecological and human health exposure assessments. Information gained from uptake studies is also important for environmental fate and effects modeling.

The current study showed that ClO_4^- was absorbed, eliminated and distributed among tissues quickly. Rapid elimination of ClO_4^- in both species was observed when the animals were transferred to clean water, with half-lives of ClO_4^- in both species of less than 1 day. Mosquitofish had lower elimination rate and higher half-life than any tissue in the catfish. This fact could be considered as the effect of body size on elimination of ClO_4^- between two species because it has been reported that elimination of water-soluble chemicals in fish is affected by allometric (size) relationship (Newman, 1995). Alternatively, this could be due to species-specific elimination mechanisms. In channel catfish, the head showed the highest uptake ($K_1 = 0.26$) of ClO_4^- .

Many water-soluble xenobiotics are taken into the body by passive diffusion through semi-permeable membranes such as gills, lining of the mouth, or gastrointestinal tract. Fish gills are especially vulnerable to foreign chemicals because their design maximizes diffusion (Spacie and Hamelink, 1999). Due to the high water solubility of ClO_4^- , the respiratory surfaces, gills, of the fish can be the main routes of ClO_4^- uptake (Evans, 2002; Marshall, 2002). Because the body burden of ClO_4^- in gills was relatively higher among tissues in this study, the main uptake of ClO_4^- via gills might play an important role in the uptake to the organs through blood system, resulting in high uptake rates in head, liver, and G-I tract. Further examination of the concentration of ClO_4^- in the blood is needed to test this assumption. For future studies, it would be important to distinguish between the primary routes of elimination in fish, because the route can be affected differently by temperature, tissue damage, pre-exposure to toxicants or the presence of competing chemicals.

The high uptake rate of ClO_4^- in head ($K_1 = 0.26 \text{ day}^{-1}$) and GI tract (0.16 day^{-1}) and low uptake rate in fillet (0.07 day^{-1}) are similar to those of Yu et al., (2002) who investigated the uptake of $^{36}\text{ClO}_4^-$ (3.3 mg/kg) over a 48 hour period in rat tissue. These authors reported that uptake of ClO_4^- showed the highest concentration in thyroid and GI tract, while the lowest $^{36}\text{ClO}_4^-$ concentration was detected in muscle. Both the head and liver of catfish had the highest uptake rate, but perchlorate concentration was more easily eliminated from the head ($K_2 = 1.22 \text{ day}^{-1}$; $T_{1/2} = 0.57 \text{ day}$) than liver (0.79 day^{-1} ; 0.88 day). This pattern was also consistent with the findings at Yu et al., (2002), and may be related to the fact that the liver is a principal organ of detoxication and excretion of many

toxicants. In the rat, the perchlorate half-life ranged from 8 to 20 hour (Wolff, 1998). Tissue clearance of ClO_4^- in catfish was slower compared to rats when the chemical was given (Yu et al., 2002). Although species, concentration and uptake period of ClO_4^- were different between two investigations, the trend of elimination was similar, low half-life in fillet and high in head, liver and G-I tract. Compared to rats, the longer half-life in fish is probably the result of lower metabolic rate in fish compared to rats.

There was also a high degree of variability in elimination of perchlorate between individuals. For example, at day 20 of elimination, a few catfish had detectable levels of perchlorate in their tissues, while most others did not. If perchlorate exposure in natural water bodies is episodic, then fish in the wild may experience periods of rapid uptake when perchlorate concentrations in the water are high, followed by a depuration period when perchlorate levels drop. Such a pattern, when combined with high inter-individual variability in elimination rates, may explain the findings that perchlorate concentrations in field-caught fish are higher than in the water, but perchlorate is usually only detected in a few individuals of each species.

In conclusion, results from the current study indicated that perchlorate is rapidly eliminated from channel catfish and mosquitofish tissue and the elimination rate constants differ between the species and between tissue types. Perchlorate kinetics in thyroid and different tissues have well been investigated in mammals, to date and to the best of our knowledge, no study has been conducted to determine the elimination of perchlorate in teleost fish. These results may be critical and used to develop models of fate, effects, and transport of ClO_4^- in natural water systems, as well as to assess ecological risk in a contaminated ecosystem.

5.4.1.4 Pharmacokinetic/Population Modeling of Perchlorate in Fish

5.4.1.4.1 Introduction

Predicting the effects of perchlorate on individual animals, much less populations of any wildlife species is a daunting task. Mathematical modeling and computer simulation may be the only possible way to gain an understanding of the dynamics of perchlorate exposure and effects in the environment. We developed a model to assist in the estimation of risk to fish populations in Lakes Belton and Waco from exposure to perchlorate. Computer simulations were conducted of the effects of perchlorate on fish populations in Lake Belton and Lake Waco in the Bosque and Leon Rivers Watersheds in Central Texas.

5.4.1.4.2 Methodology

A physiologically based toxicokinetic (PBTK) model was developed to simulate the movement of perchlorate within the channel catfish. Contaminant movement was governed by a series of mass-balanced differential equation programmed in Matlab®. Model compartments and blood flow can be seen in **Figure 5-151**. General equations used in the model were taken from PBTKs developed by Nichols et al. for rainbow trout (1990 and 1991) and channel catfish (1993). The rate of change for each of the seven compartments (skin, kidney, muscle, GI tract, liver, fat, and thyroid) at individual time

steps is obtained by solving the differential equations simultaneously using numerical integration. Fish gill physiology was kept biologically accurate by accounting for countercurrent chemical flux, including both flow and diffusion limitations (Erickson and McKim, 1990b). Flow-limited distribution was assumed i.e. chemical equilibrium existed between the tissues and blood leaving the compartment. Additionally portal blood flow was incorporated into the kidney and liver from poorly perfused tissue and richly perfused tissue respectively. Portal blood flow to the kidney was set as 60% of blood flow to skin and muscle compartments, with portal flow to the liver equal to blood flow to the GI tract (Nichols et al., 1990).

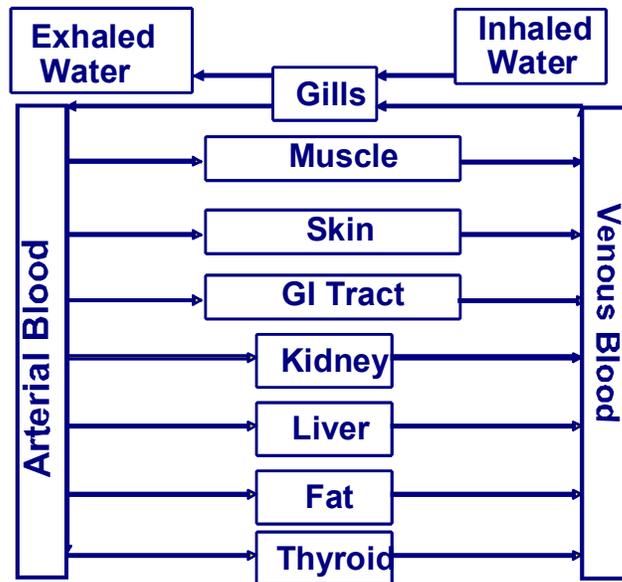


Figure 5-151
Flow Diagram of the PBTK Model for Perchlorate Inhalation in Fish

Physiological parameters for blood flow and cardiac output in the channel catfish were obtained from existing literature (Erickson and McKim, 1990a; Erickson and McKim, 1990b; Hughes, 1984; Nichols et al., 1990; 1993). Blood flow, cardiac output, and ventilation parameters were based on a 1 kg adult catfish, significantly larger than the 60g fishes used for tissue volumes and calibration. This difference was accounted for by scaling tissue blood flow by body weight to the 0.75 power. Tissue masses were measured in undosed juvenile channel catfish (Bradford, 2002), with their averages used to determine their fractional mass (**Table 5-40**).

Table 5-40
Physiological Parameters Used in the PBTK Model for Channel Catfish

Weight (kilograms)	BW = 0.0605
Oxygen Consumption Rate (mg/h) ^a	VO = 71.9
Oxygen Uptake efficiency (%) ^a	UE = 48.9
Ventilation volume (l/h) ^a	QV = 17.7
Cardiac output (l/h per kg) ^a	CO = 2.5
Tissue group volumes (%)^{b,c}	
Fractional volume of kidney	VK = 0.0074
Fractional volume of liver	VL = 0.019
Fractional volume of thyroid	VT = 0.0941
Fractional volume of GI tract	VG = 0.0795
Fractional volume of skin	VS = 0.03971
Fractional volume of muscle	VM = 0.17785
Fractional volume of fat	VF = 0.066
Arterial blood flow to tissues (%)^d	
Fractional blood flow to kidney	QK = 0.06
Fractional blood flow to liver	QL = 0.036
Fractional blood flow to GI tract	QG = 0.16
Fractional blood flow to thyroid	QT = 0.1908
Fractional blood flow to skin	QS = 0.0805
Fractional blood flow to muscle	QM = 0.3606
Fractional blood flow to fat	QF = 0.112

^aValues given are for a 1 kg catfish.

^bTissue volumes are calculated as a product of the fractional volume and body weight(l).

^cFractional volumes are calculated as the average of measured tissue mass for catfish used in calibration.

^dTissue blood flow (l/h) calculated as follows: $q_i = Q_i \cdot (CO \cdot BW^{0.75})$

^eAll partitioning coefficients (P_{ij}) were derived through calibration.

Route of exposure for perchlorate in the PBTK model was uptake through inspired water, which was simulated by using an equation for chemical flux at the gills (Erickson and McKim, 1990b). This method accounts for counter-current exchange with flows separated by a diffusion barrier made up of the gill epithelium and stagnant boundary layers in adjacent blood and water channels (Nichols et al., 1993). For purposes of this model, diffusion resistances were considered negligible in flow channels. By integrating diffusive flux on the gill surface, we arrive at an equation that relates total flux (F_g in $\mu\text{g/h}$) to an exchange coefficient and the difference in chemical activities between the venous blood and inspired water:

$$F_g = k_x \times \left(C_{insp} - \frac{C_{ven}}{P_{bw}} \right)$$

Exchange coefficient (k_x) is determined by the chemical capacities of water (k_w) and blood (k_b) flowing to perfused gill lamellae, and the average resistance to chemical diffusion (k_d), each parameter's sensitivity can fluctuate based on the chemical and organism (Erickson and McKim, 1990b):

$$k_x = \frac{\frac{e^{-k_d/k_b} - e^{-k_d/k_w}}{k_w} - \frac{e^{-k_d/k_b} - e^{-k_d/k_w}}{k_w}}{\frac{e^{-k_d/k_b} - e^{-k_d/k_w}}{k_w} - \frac{e^{-k_d/k_b} - e^{-k_d/k_w}}{k_w}}$$

Chemical capacity of water flowing to the perfused gill lamellae is equal to the effective respiratory volume (Q_w), with chemical capacity calculated as follows:

$$k_b = Q_c \times P_{bw}$$

Diffusional resistance was calculated with the following equation:

$$k_d = D \times \frac{A}{T}$$

where: D = molecular diffusivity (m^2/h)
 A = total lamellar gill area (m^2)
 T = diffusion barrier thickness (m)

Because of limited data on catfish gill morphometry it was necessary to use values for total gill area and epithelial thickness reported for other fish species exhibiting intermediate levels of activity (Hughes, 1984).

Total lamellar gill area was scaled to body weight as follows:

$$A = \frac{(600 \times BW^{0.8})}{1000}$$

Erickson and McKim (1990b) calculated diffusion barrier thickness as the sum of the thickness of the epithelial layer plus one-third the combined thickness of the water and blood layers. An epithelial thickness of 4 μm was used by Nichols et al. (1993) as an average of the values reported by Hughes (1984), resulting in a total diffusion barrier of 12 μm .

Molecular diffusivity of perchlorate in water was calculated using an equation developed by Wilke and Chang (1955), and estimated as 0.0561 cm^2/s :

$$D_{BW} = \frac{7.4 \times 10^{-8} (\rho_w M_w)^{1/2} T}{\eta_w V_B^{0.6}}$$

where ϕ_W = solvent association factor
 MW = molecular weight of water (g/mol)
 T = temperature (K)
 η_W = viscosity of water (cP)
 VB = molar volume of solute B (cm³/mol)

Reduced permeability across the gill epithelia was accounted for by multiplying the molecular diffusivity by 0.75 to obtain combined diffusivity (Erickson and McKim, 1990b). See **Table 5-41** for an explanation of abbreviations and model parameter symbols.

Table 5-41
Abbreviations and Symbols Used

F^G = flux of perchlorate across the gills, mg·kg ⁻¹ ·h ⁻¹
k_X^G = exchange coefficient
f_W = ratio of free chemical in exposure water to total concentration
f_B = ratio of free to total perchlorate in blood
$C_W^{aff,G}$ = total concentration of perchlorate in exposure water, mg·kg ⁻¹
$C_B^{aff,G}$ = concentration of perchlorate in the blood afferent to the gills, mg·kg ⁻¹
$C_B^{eff,G}$ = concentration of perchlorate in the blood efferent to the gills, mg·kg ⁻¹
$C_B^{eff,i}$ = concentration of perchlorate in the blood efferent to the tissue compartment, mg·kg ⁻¹
Q_B^i = blood flow rate from the tissue, L·h ⁻¹
Q_B^G = total cardiac output, L·h ⁻¹ ·kg
A^i = amount of perchlorate in the tissue, mg
V^i = volume the tissue compartment, kg
K_B^i = tissue/blood partitioning coefficient
$\frac{dA^i}{dt}$ = rate of change in perchlorate concentration in the tissue compartment, mg·kg ⁻¹ ·h ⁻¹

Water intake (dose) is governed by the gill flux equation below :

$$F^G = k_X^G (f_W C_W^{aff,G} - f_B C_B^{aff,G})$$

The concentration of perchlorate in the blood afferent to the gills, also known as the venous blood, was calculated as follows:

$$C_B^{aff,G} = \frac{\sum(C_B^{eff,i} * Q_B^i)}{Q_B^G}$$

The concentration of perchlorate in blood efferent to each tissue compartment was calculated as follows:

$$C_B^{eff,i} = \frac{\left(\frac{A^i}{V^i} \right)}{K_B^i}$$

The rate of change in perchlorate concentration for each tissue compartment is defined by the differential equation:

$$\frac{dA^i}{dt} = Q_B^i (C_B^{eff,G} - C_B^{eff,i})$$

Finally, the concentration of perchlorate in the blood efferent to the gills was calculated as follows:

$$C_B^{eff,G} = C_B^{aff,G} + \frac{F^G}{Q_B^G}$$

For our purposes the PBTK model was calibrated by altering the partitioning coefficients, the only parameters that were not taken from the published literature or directly measured. The calibration data were taken from an 11-day elimination study in channel catfish (Theodorakis et al., 2003). Juvenile channel catfish were exposed to 84 ± 4 ppm perchlorate for two days, followed by movement to a clean tank for an 11-day elimination period. By fitting the model to the elimination data as well as the uptake data we were able to account for the lack of a urinary excretion term (**Figure 5-152** through **Figure 5-158**).

Model calibration was tested by calculating the root mean squared (RMS) for the gill, GI, muscle, liver and thyroid compartments and conducting a sensitivity analysis. The purpose of using the RMS was to create a quantifiable measure of calibration “goodness” while accounting for simulated values greater than or less than the calibration values. A mean error calculation would not differentiate between the two, potentially leading to a false low value that was achieved by positive and negative errors canceling each other out.

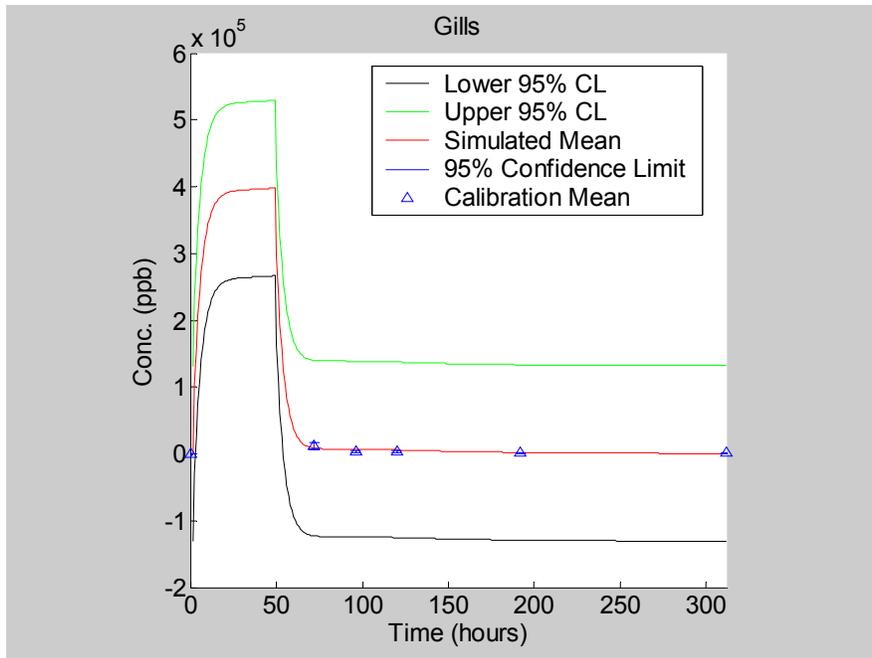


Figure 5-152
Calibration Curve for Gill Compartment Using Elimination Data

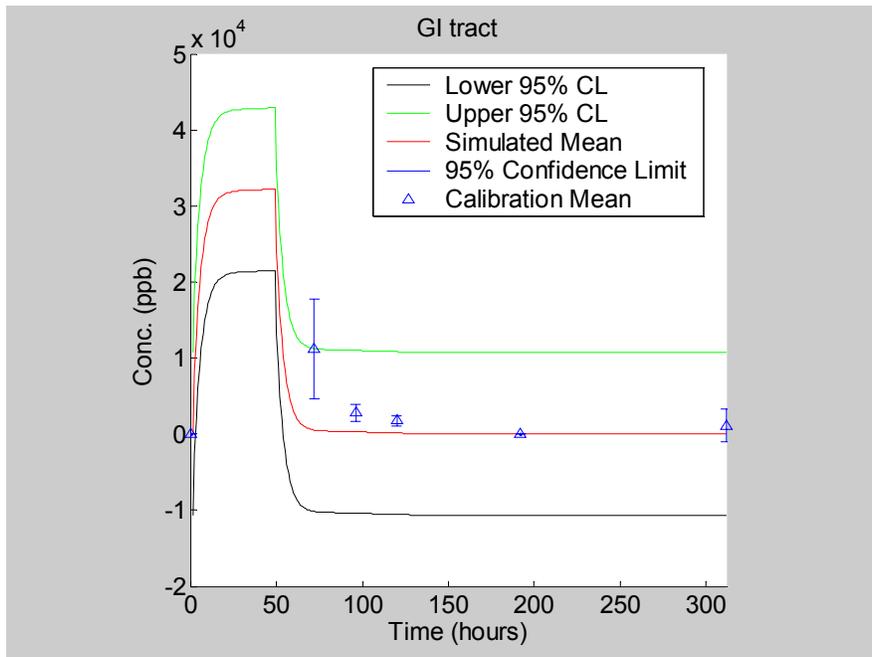


Figure 5-153
Calibration Curve for GI Compartment Using Elimination Data

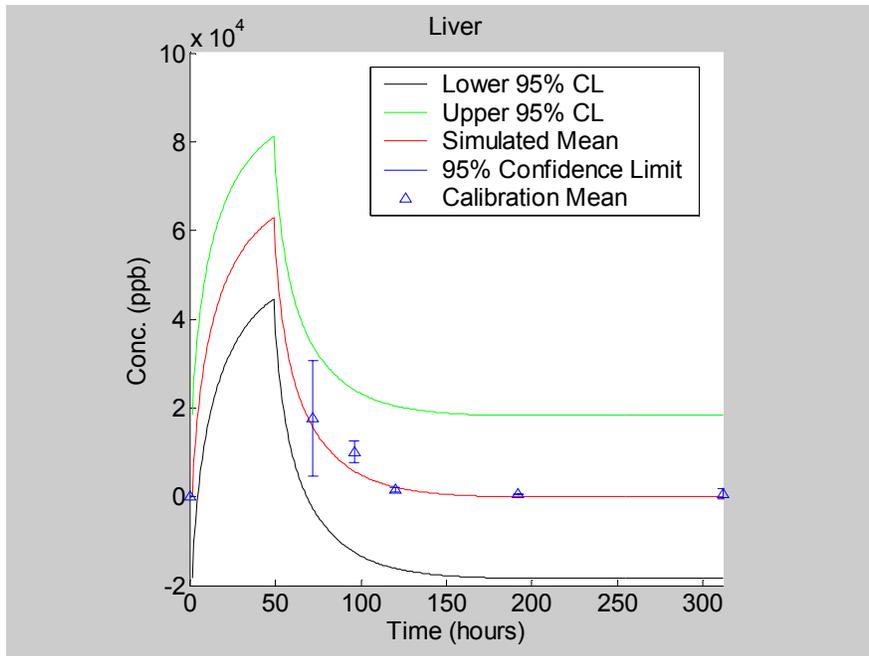


Figure 5-154
Calibration Curve for Liver Compartment Using Elimination Data

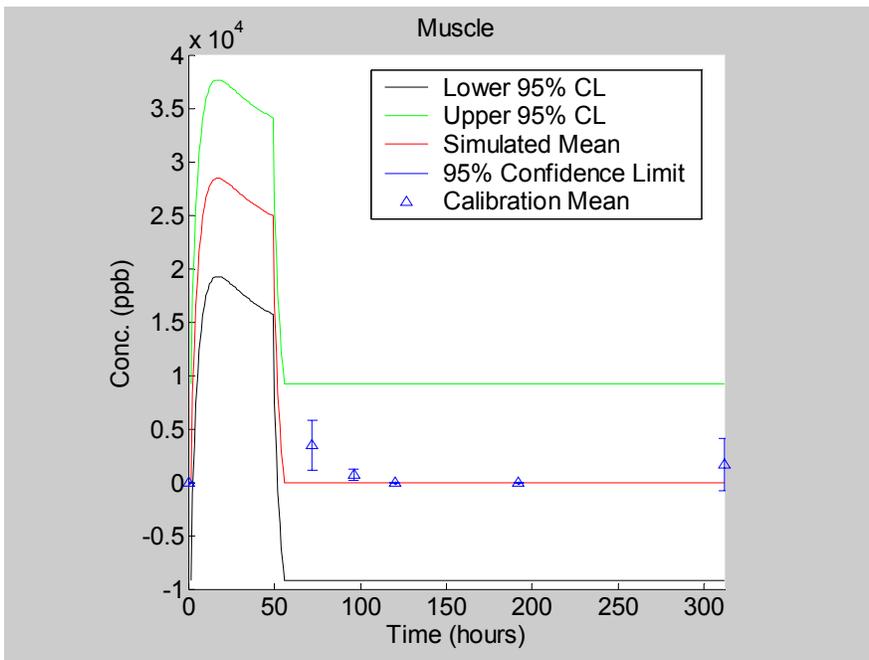


Figure 5-155
Calibration Curve for Muscle Compartment Using Elimination Data

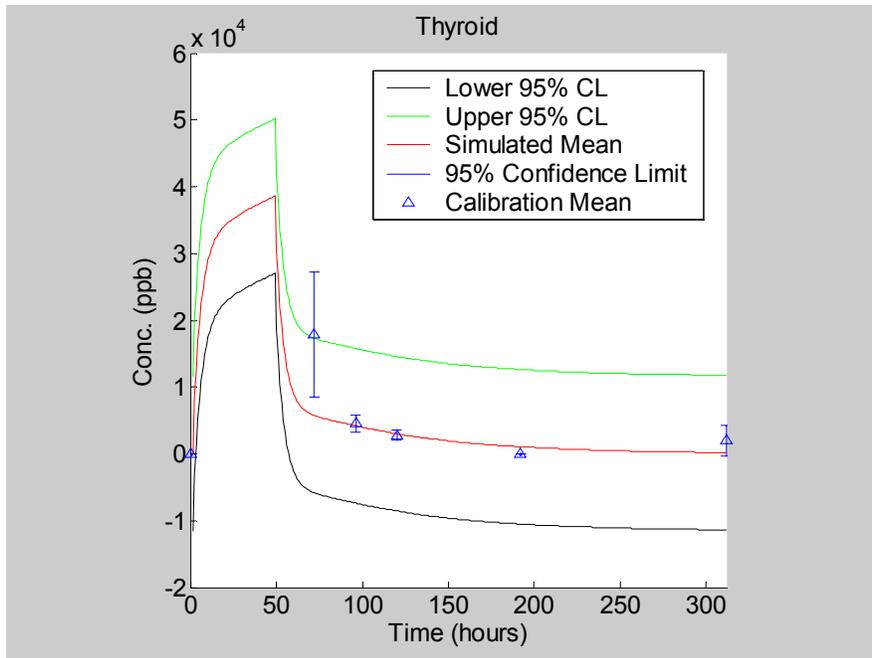


Figure 5-156
Calibration Curve for Thyroid Compartment Using Elimination Data

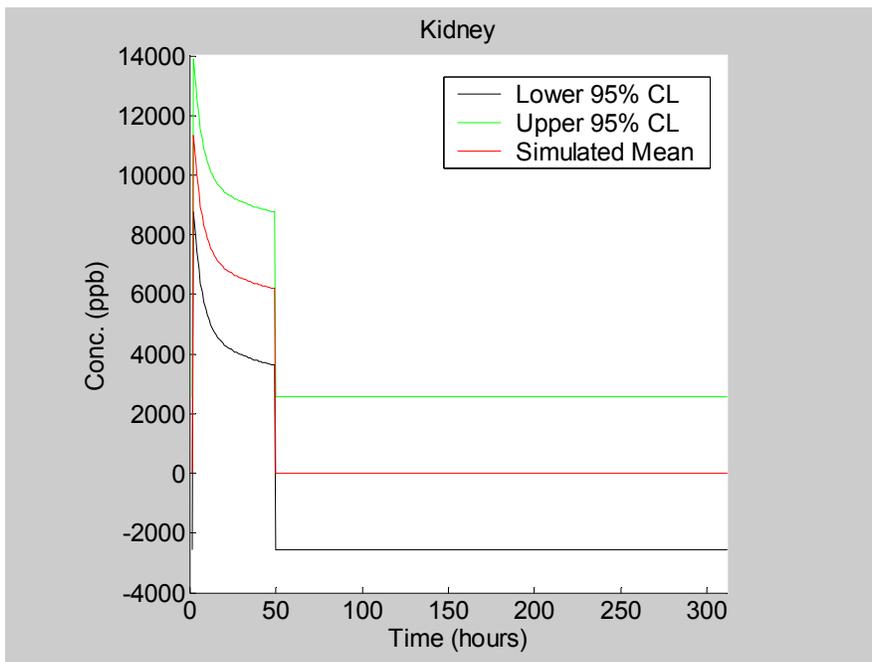


Figure 5-157
Partial Calibration Curve for Kidney Compartment Using Dosing Data

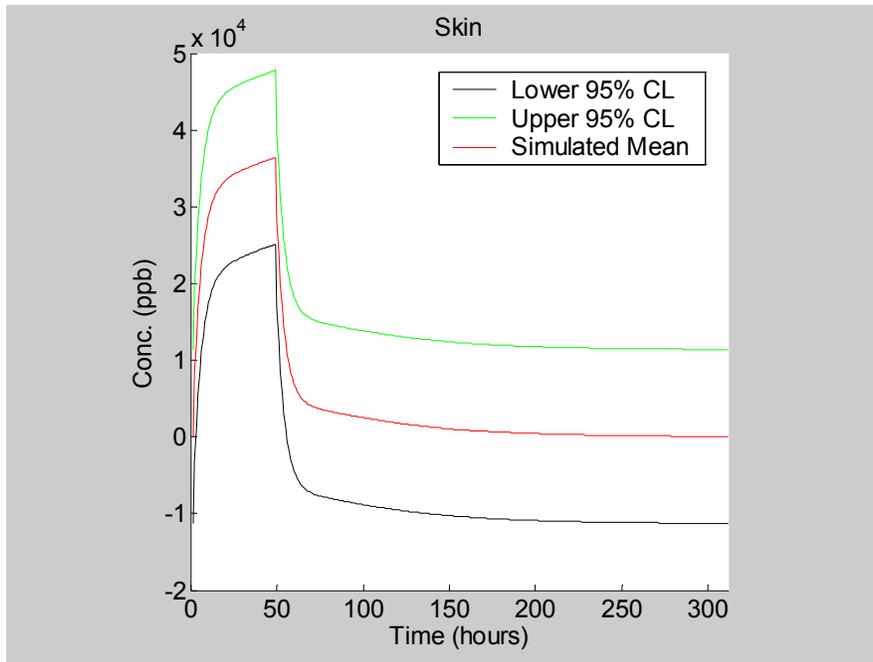


Figure 5-158
Partial Calibration Curve for Skin Compartment Using Dosing Data

For the sensitivity analysis, each partitioning coefficient was adjusted individually by $\pm 15\%$ and the parameter sensitivity recorded (**Table 5-42**). This information provides a measure of uncertainty in the model and determines how greatly this uncertainty can affect model output. Sixty-eight of the 98 permutations were sensitive, indicating the need for accurate partitioning coefficients. Varying the coefficients by $\pm 15\%$ is a very conservative range since measured partitioning coefficients provide an exact number. The area of greatest sensitivity in model output is as tissue concentration approaches equilibrium and complete elimination in the first 72 hours. This should be expected because of the very steep slope (**Figure 5-159**). A compartment was considered sensitive to a parameter adjustment if its sensitivity coefficient was greater than one:

$$S = \frac{\frac{(X_a - X_n)}{X_n}}{\frac{(P_a - P_n)}{P_n}}$$

where X_a = adjusted variable
 X_n = nominal variable
 P_a = adjusted parameter value
 P_n = nominal parameter value

Table 5-42
Variable Sensitivity to Parameter Adjustment (+ Sensitive, - Sensitive)

	Gill	GI	Kidney	Liver	Muscle	Skin	Thyroid
kbw -15%	+	+	+	+	+	+	+
kbw +15%	+	+	-	+	+	+	-
kg -15%	+	+	+	+	+	+	+
kg +15%	+	+	-	+	+	-	-
kk -15%	+	+	+	+	+	+	+
kk +15%	-	+	-	+	-	-	-
kl -15%	+	+	+	+	+	+	+
kl +15%	+	-	-	+	-	-	-
km -15%	+	+	-	-	+	-	-
km +15%	+	+	+	+	+	+	+
ks -15%	+	-	-	-	-	+	-
ks +15%	+	+	+	+	+	+	+
kt -15%	+	+	+	+	+	+	+
kt +15%	+	-	-	-	-	-	+

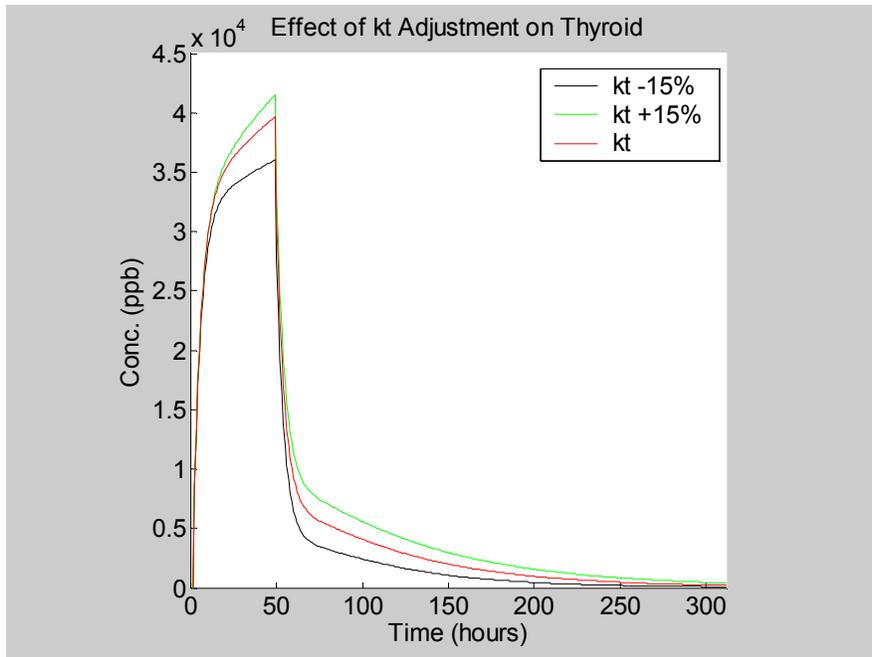


Figure 5-159
Effect of Increasing or Decreasing Thyroid-Blood Partitioning Coefficient (kt) by 15% on Concentration of Perchlorate in the Thyroid Compartment

Thyroid Model Description. A six-compartment model of combined T₃ and T₄ kinetics originally developed for mammals (DiStefano, 1986; Bianchi et al., 1987; Pilo et al., 1990; Hershman et al., 1986) and subsequently applied to rainbow trout (Sefkow et al., 1996) was used to simulate the secretion of T₃ and T₄ in channel catfish, as well as the

impacts of perchlorate on secretion rates. The six compartments represented included: T₄ slow exchange tissue pool, T₄ rapid exchange tissue pool, T₄ plasma pool, T₃ slow exchange tissue pool, T₃ rapid exchange tissue pool, and T₃ plasma pool. Skeletal muscle makes up the majority of the slow tissue pool. The rapid tissue pool consists primarily of the liver, but also the kidney and gill, all of which possess deiodinating capacity (MacLatchy and Eales, 1992; Eales and Brown, 1993). While total T₃ production is lower in the rapid pool, compared to the slow pool, its rapid efflux (12x greater) makes it an essential component in the acute adjustment of plasma T₃ levels (Sefkow et al., 1996).

Model Parameters and Equations. Parameters in the model were either uniquely identifiable (**Table 5-43**) or determined within a range using the method of interval identifiability analysis (DiStefano, 1983). Interval identifiability analysis uses estimates of identifiable parameter combinations to determine bounded intervals. An example of this is:

$$k_{21}^{\min} \equiv \frac{\gamma_2 - \xi_2 / k_{55}}{-k_{22}} \leq k_{21} \leq -k_{11} - k_{31}^{\min} \equiv k_{21}^{\max}$$

Feng and DiStefano (1995) used interval analysis to obtain finite bounds for all combinations of kij. These relationships in turn were used to bound the unidentifiable steady-state compartment masses and percentage of T₃ converted to T₄ (Sefkow et al., 1996).

Table 5-43
Parameters Used in Thyroid Model

k_{ij} = Flow rate to compartment i from compartment j (min^{-1})
Q_i = Hormone concentration in compartment i (ng/ml)
$[T_4]_p$ = Endogenous T ₄ plasma concentration at steady state (ng/ml)
$[T_3]_p$ = Endogenous T ₃ plasma concentration at steady state (ng/ml)
V_p = Plasma volume (ml)
SR3 = T ₃ secretion rate ($\text{ng/hr} \cdot 100\text{g BW}$)
SR4 = T ₄ secretion rate ($\text{ng/hr} \cdot 100\text{g BW}$)
CR_{4-3}^{slow} = T ₄ to T ₃ conversion rate in the slow compartment (ng/hr)
CR_{4-3}^{fast} = T ₄ to T ₃ conversion rate in the fast compartment (ng/hr)
ξ_i = uniquely identifiable parameter combination (min^{-3})
γ_i = uniquely identifiable parameter combination (min^{-2})

Only the plasma compartments have uniquely identifiable steady-state masses. The remaining compartments are defined within a given interval based on the minimum and maximum values of a unique unidentifiable parameter within each compartment. Steady-state equations were developed by Sefkow et al. (1996) based on data from fasted rainbow trout injected with radiolabeled T₃ and T₄.

Steady-state mass in T₄ plasma compartment (Q₁):

$$Q_1 = [T_4]_p V_p$$

Steady-state mass in T₃ plasma compartment (Q₄):

$$Q_4 = [T_3]_p V_p$$

Steady-state mass in T₄ rapid exchange compartment (Q₂):

$$Q_2 = \left(\frac{Q_1}{-k_{22}} \right) k_{21}$$

Steady-state mass in T₃ rapid exchange compartment (Q₅):

$$Q_5 = \left(\frac{Q_4}{-k_{55}} + \frac{\xi_2 Q_1}{\gamma_5 k_{22} k_{55}} \right) k_{54}$$

Steady-state mass in T₄ slow exchange compartment (Q₃):

$$Q_3 = \left(\frac{Q_1}{-k_{33}} \right) k_{31}$$

Steady-state mass in T₃ slow exchange compartment (Q₆):

$$Q_6 = \left(\frac{Q_4}{-k_{66}} + \frac{\xi_3 Q_1}{\gamma_6 k_{33} k_{66}} \right) k_{64}$$

Secretion rates were found by assuming a rate of change equal to zero and solving for exogenous constant-rate inputs into the plasma compartments:

$$SR_4 = \left(-k_{11} + \frac{\gamma_2}{k_{22}} + \frac{\gamma_3}{k_{33}} \right) Q_1$$

$$SR_3 = \left(-k_{44} + \frac{\gamma_5}{k_{55}} + \frac{\gamma_6}{k_{66}} \right) Q_4 - \left(\frac{\xi_2}{k_{22} k_{55}} + \frac{\xi_3}{k_{33} k_{66}} \right) Q_1$$

T₄ to T₃ conversion rates in the slow and fast pool compartments were solved using a similar approach:

$$CR_{4-3}^{slow} = k_{63}Q_3 = \frac{\xi_3 k_{64}}{-k_{33}\gamma_6} Q_1$$

$$CR_{4-3}^{fast} = k_{52}Q_2 = \frac{\xi_2 k_{54}}{-k_{22}\gamma_5} Q_1$$

Model Calibration. Since the steady state values reported by Sefkow et al. (1996) were calculated from laboratory data for rainbow trout, it was important to make adjustments to hormone levels to better represent channel catfish. It is also important to note that the steady-state values are not based on a mass-balance model as used in this research. Compared to the T₃ and T₄ levels of 7.1 x 10⁻⁴ ng/mL and 1.04 x 10⁻³ ng/mL respectively, reported by Sefkow et al (1996), Gaylord et al (2001) reported plasma T₃ levels of ~7.6-13.3 ng/mL and T₄ levels of ~2.3-4 ng/mL in channel catfish. Additionally the regular fluctuations in hormone levels within fish make a “steady-state” value misleading. T₃ levels are relatively stable with an average of 0.4 ± 0.1 peaks/24 hour period significantly greater than baseline with an average amplitude of 1.7 ± 0.2 ng/mL. T₄, however, exhibited 2.5 ± 0.2 peaks/24 hour period with an average amplitude of 3.0 ± 0.4 ng/mL (Gomez et al., 1997).

Initially, the model had to be run long enough for the compartments to equilibrate from a starting concentration of 0 ppm. The number of time steps (hours) necessary to achieve a baseline value ranged from 500 to 1000 depending on the compartment. Once our baseline (steady-state) had been achieved we could proceed with calibration. The first step in model calibration for the thyroid model was to compare the mass-balance model results to those reported by Sefkow et al (1996), as stated previously the values would not be expected to be similar because of the different techniques utilized. Instead the percentage of hormone in each compartment was used for comparison, as mass-balance ratios should remain constant. All compartments were within 1% of the percentages as reported in the literature, indicating good model structure (**Table 5-44**). Plasma T₃ and T₄ compartments were then adjusted, keeping the same percentage distribution with other compartments, to meet levels reported by Gaylord et al. (2001) in channel catfish (**Figure 5-160** through **Figure 5-165**). Alteration of plasma T₃ and T₄ levels automatically adjusts hormone concentrations in the remaining compartments based on mass-balance.

Table 5-44
Comparison of Simulated and Reported Values for Hormone Distribution in Model Compartments

	Sefkow et al. (1996)	Simulated Percentage
Plasma T ₃	13.7%	13.7%
Fast Pool T ₃	12.6%	12.2%
Slow Pool T ₃	73.7%	74.7%
Plasma T ₄	18.5%	17.8%
Fast Pool T ₄	16.2%	15.9%
Slow Pool T ₄	65.3%	66.3%

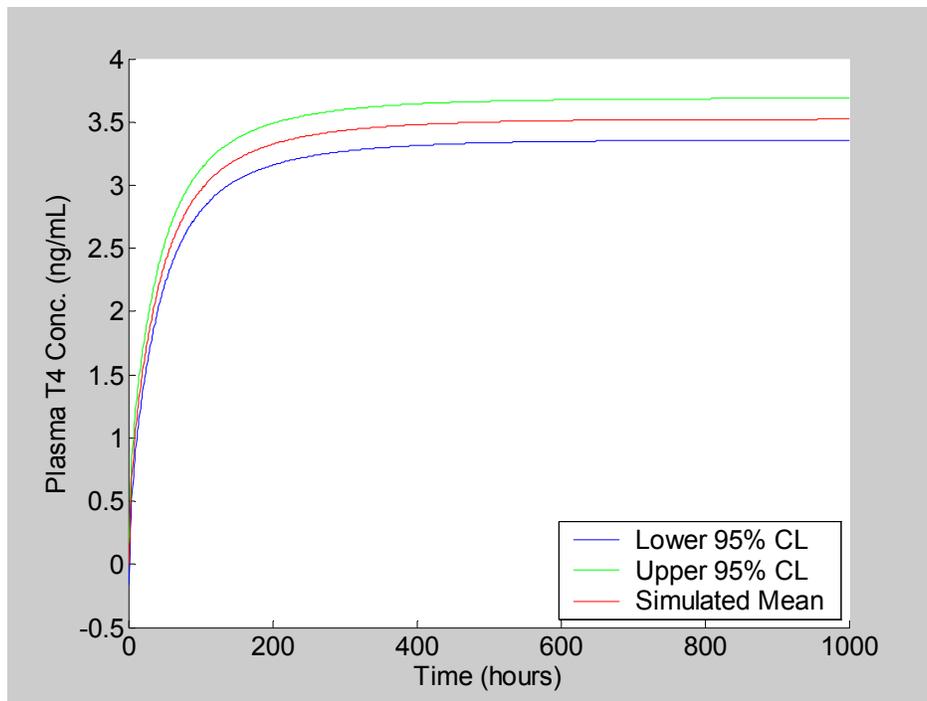


Figure 5-160
Calibrated T₄ Plasma Compartment Curve

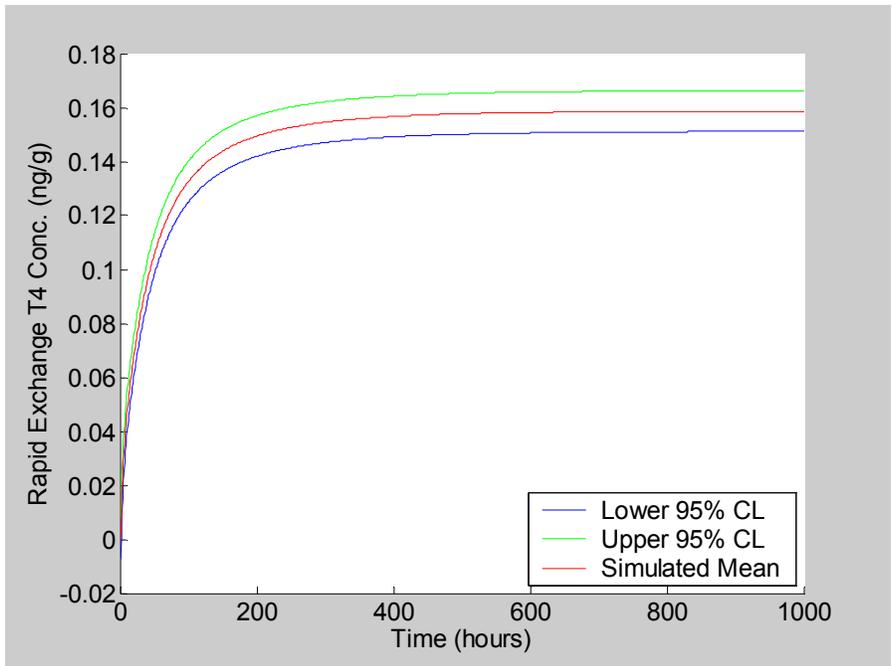


Figure 5-161
Calibrated T₄ Rapid Exchange Compartment Curve

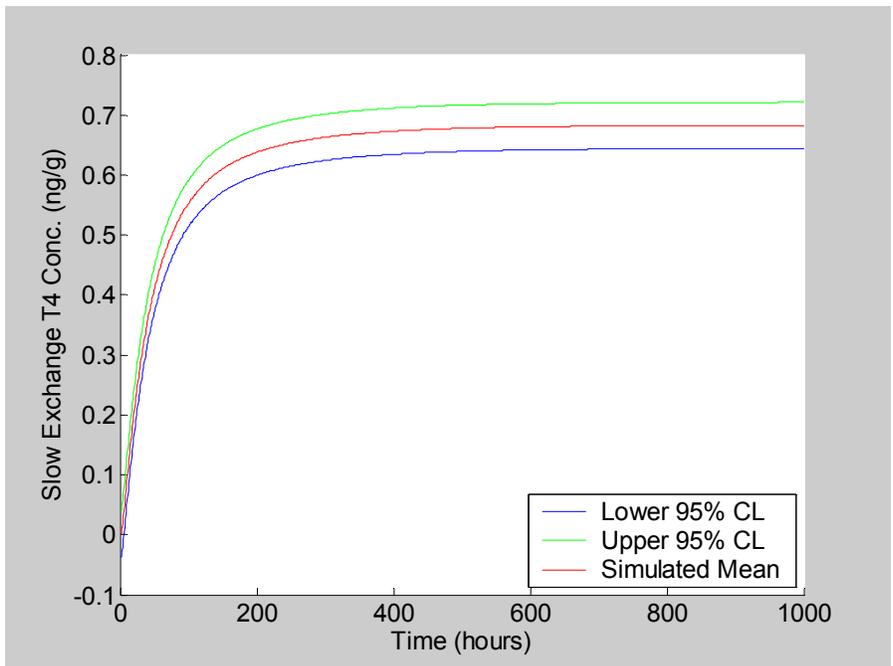


Figure 5-162
Calibrated T₄ Slow Exchange Compartment Curve

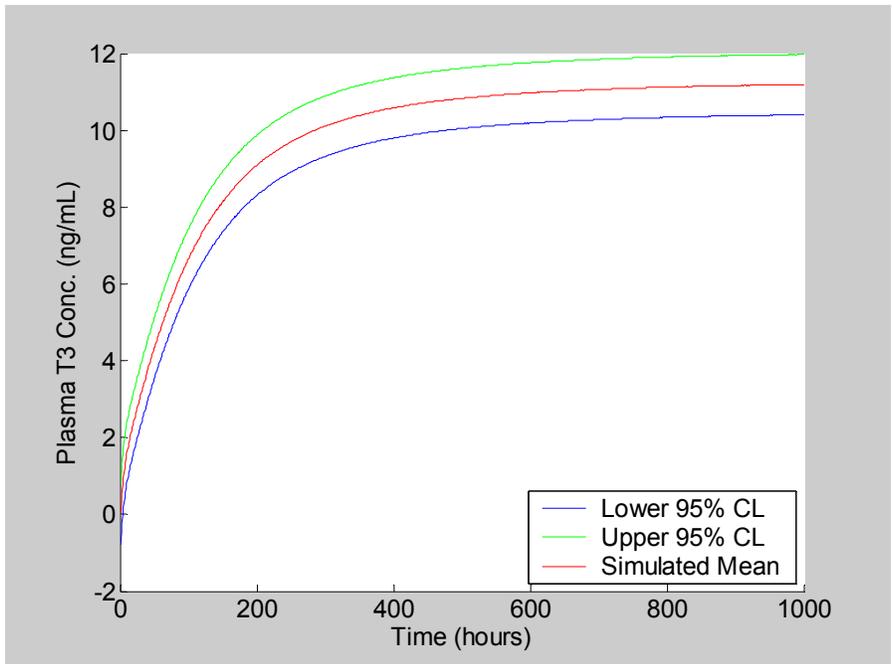


Figure 5-163
Calibrated T₃ Plasma Compartment Curve

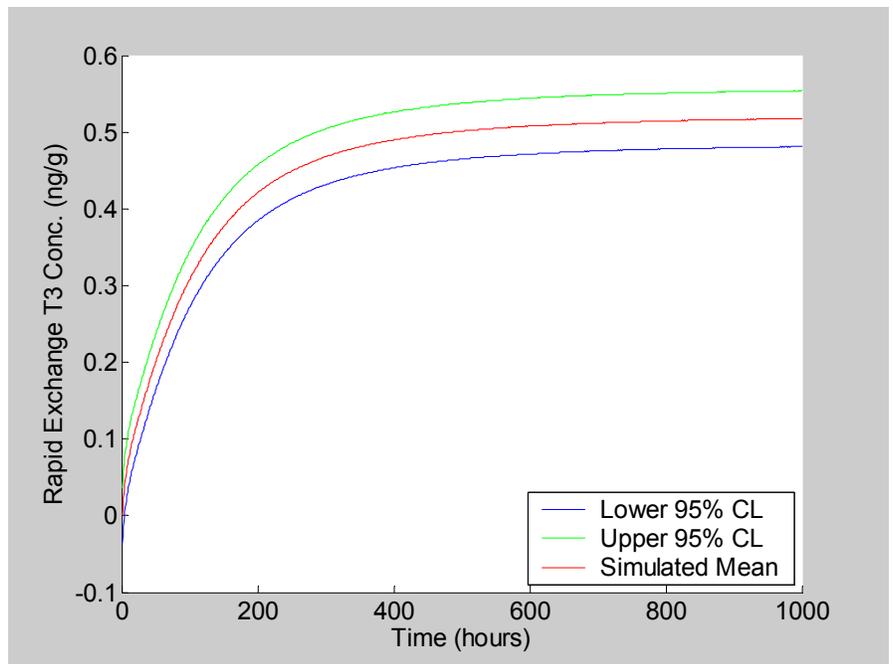


Figure 5-164
Calibrated T₃ Rapid Exchange Compartment Curve

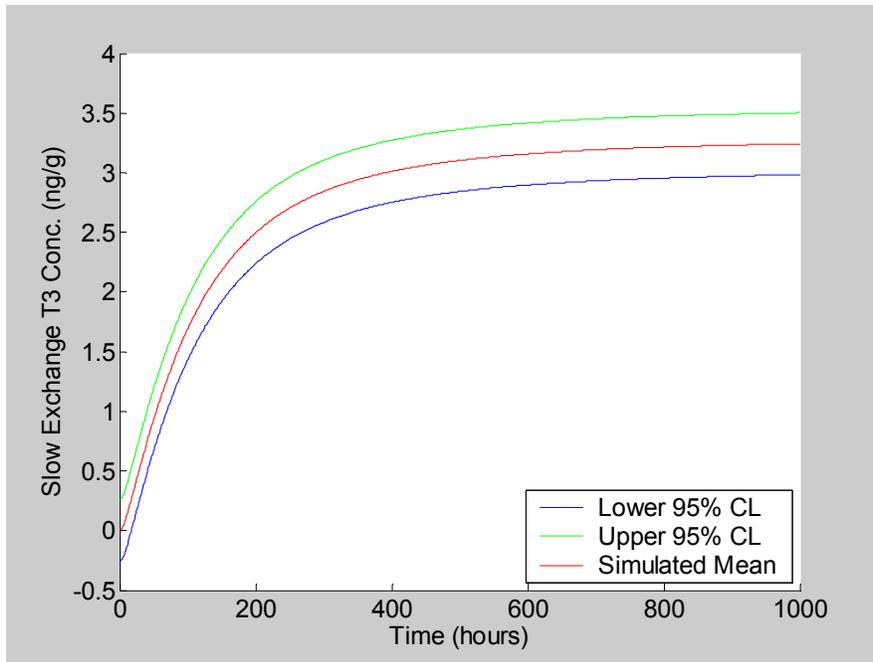


Figure 5-165
Calibrated T₃ Slow Exchange Compartment Curve

Hormone Inhibition. Hormone inhibition by perchlorate was calculated from data on mosquitofish dosed with sodium perchlorate for 2, 10, or 30 days at doses of 0, 0.1, 1, 10, 100, and 100 mg/L (ppm) (Bradford, 2002). Whole body T₄ concentrations were determined by radioimmunoassay for pooled groups of fish. A regression curve was fit to the data to derive the inhibition equation based on the concentration of perchlorate in the thyroid tissue (DT):

$$APfactor = 0.686 - 0.1047 \times \log(DT)$$

This equation was in turn applied to the three T₄ compartments in the model (plasma, rapid exchange and slow exchange) through adjustment of the T₄ secretion rate term. While this was the only data available for perchlorate related hormone inhibition in fish, it is not robust enough to draw a definitive conclusion as to the inhibition properties of perchlorate. A sensitivity analysis performed on the inhibition term (APfactor), as described above, identified all compartments as insensitive to an increase or decrease in the APfactor of 15% (Figure 5-166).

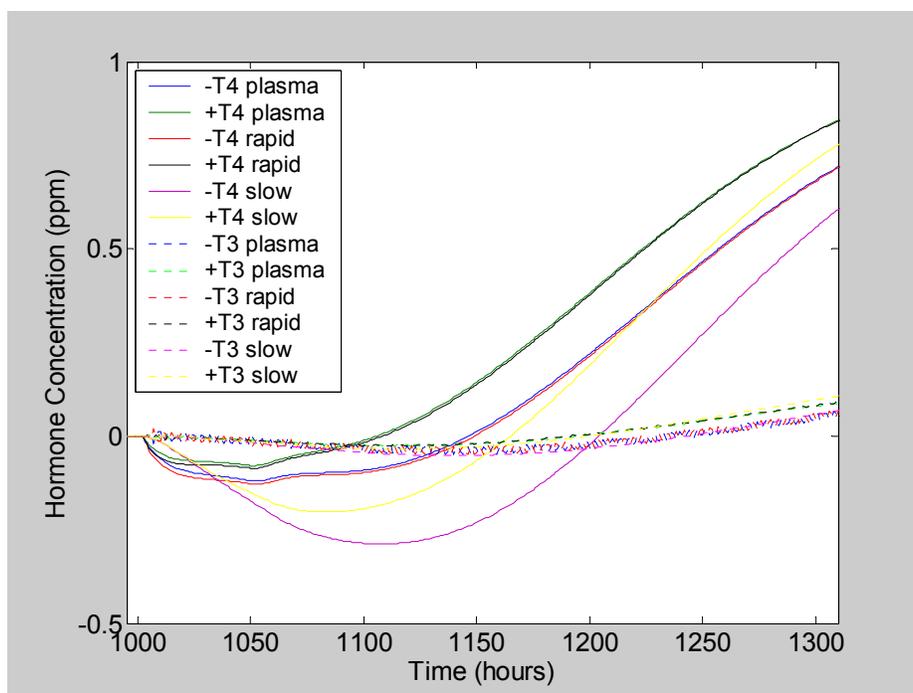


Figure 5-166
Sensitivity Analysis for Thyroid Hormone Compartments Based on $\pm 15\%$ Change to AP Factor

5.4.1.4.3 Data

5.4.1.4.3.1 Exposure Scenario

A “worst-case” scenario was developed, in which individual catfish were exposed to the highest measured field water concentrations until their tissues reached equilibrium. Field values varied widely, with the highest reported value being 540 ppb in S Creek at Highway 317 (T15) (Todd Anderson, personal communication). Most tissue compartments took approximately 120 hours to equilibrate, with the gill and thyroid compartment taking 250 and 500 hours respectively (**Figure 5-167**, **Figure 5-168**). If we assume that 540 ppb is the highest level any fish is exposed to then all measured field tissue concentrations should be less than or equal to those simulated. The highest measured muscle concentration, based on tissue wet weight, was 60 ppb. Simulated muscle concentrations were ~150 ppb, indicating model validity. Since thyroid tissue cannot be collected in fish, the head was used as an approximate measure. Field measurements had a high wet weight concentration of 850 ppb compared to an initial simulated 300 ppb. The model was re-calibrated with the field data resulting in a mean simulated thyroid concentration of 880 ± 120 ppb (mean $\pm 95\%$ CI) after 500 hours. The thyroid concentration data were then used in the hormone secretion model to determine the level of hormone inhibition. Perchlorate inhibition was initiated after 1000 hours to allow the hormone compartments to reach steady-state prior to insult (**Figure 5-169**). T_3 hormone levels decreased 18.7-22% and T_4 levels decreased 56.5% (**Table 5-45**).

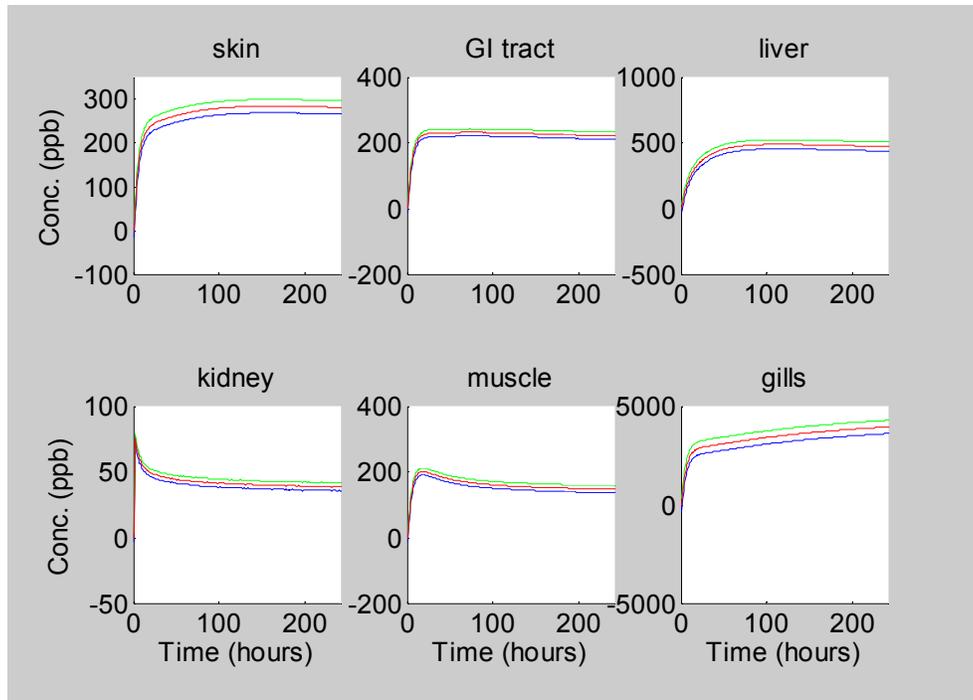


Figure 5-167
Simulated Tissue Concentrations Based on a 540 ppb Perchlorate Exposure

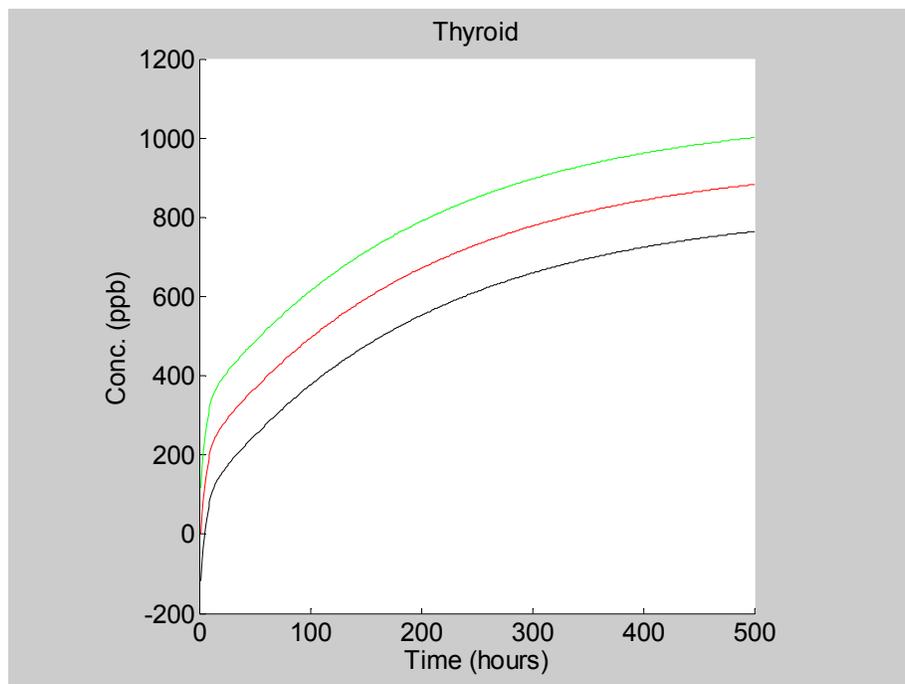


Figure 5-168
Simulated Thyroid Tissue Concentrations Based on a 540 ppb Perchlorate Exposure

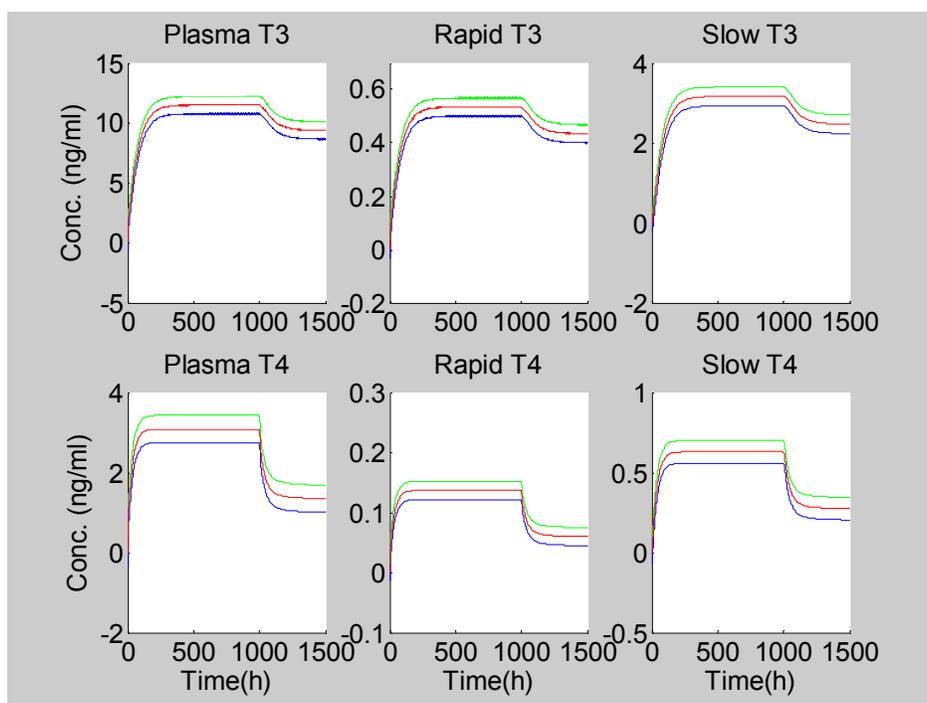


Figure 5-169
Simulated Hormone Inhibition Based on 540 ppb of Perchlorate Exposure Starting at Hour 1000

Table 5-45
Thyroid Hormone Inhibition Based on 540 ppb Perchlorate

	Steady State ng/mL	Simulated ng/mL (% decrease)
Plasma T ₃	11.5	9.3557 (18.7%)
Fast Pool T ₃	0.5347	0.4342 (18.8%)
Slow Pool T ₃	3.1605	2.4652 (22%)
Plasma T ₄	3.0908	1.3440 (56.5%)
Fast Pool T ₄	0.1373	0.0597 (56.5%)
Slow Pool T ₄	0.63	0.2741 (56.5%)

5.4.1.4.4 Discussion

The PBTK model was able to reproduce field results indicating model validity. Because of the complexity of real world systems, it is not uncommon for additional calibration of the model to occur. The limited data sets available for initial model calibration resulted in a simplification of the thyroid compartment. A singular thyroid compartment may be an oversimplification of the kinetic behavior of perchlorate. Chow and Woodbury (1970) determined that a three-compartment model (stroma, follicle, and lumen) was necessary to properly model the behavior of perchlorate in rat thyroids. It is unknown if fish would follow a similar kinetic behavior since they lack the centralized thyroid gland of

mammals. The simplification of the thyroid compartment, in conjunction with the use of 100 ppm dosing data for calibration, may have underestimated uptake at low doses.

The rapid, and substantial, decrease in hormone levels should be viewed cautiously. We do not know if channel catfish respond in the same way to perchlorate exposure as the mosquitofish used for the inhibition term. Additionally we cannot fully characterize the system without the data necessary to determine a T_3 inhibition term, hence the reason T_3 levels decreased substantially less than T_4 . At present the simulated results are only a “best guess” and should not be viewed as a definitive answer. It is also important to note that fish are very adept at altering thyroid hormone levels based on various environmental and dietary conditions. As a result of this ability, fish tend to rapidly reestablish normal hormone levels once the inhibitory condition is removed. In addition, various studies have shown that different species rapidly eliminate perchlorate and recover from exposure, once they are removed from a contaminated system.

5.4.2 Frogs

Environmental perchlorate could be a potential threat to vertebrate wildlife because it blocks iodide uptake by thyroid follicular cells. Because the mechanism of iodide uptake and thyroid hormone synthesis is essentially identical in all vertebrates, the extensive data set on perchlorate mode of action that has collected over the last 50-60 years can be used to predict potential effects in wildlife. One of the groups at greatest risk are frogs (amphibians), as they require normal thyroid hormone secretion to complete the transformation from tadpole to frog. If tadpoles do not complete this transformation they cannot reproduce. If metamorphosis is delayed, tadpoles are at risk of greater exposure to predators and pond drying. Finally, because metamorphosis is such a robust and dramatic morphological change, the effects of perchlorate can be observed using non-invasive endpoints such as limb emergence, tail resorption, or limb length.

Previous work (Goleman et al., 2002a,b; Carr et al., 2003) indicates that exposure to environmentally relevant concentrations of perchlorate delays the completion of metamorphosis and alters sex ratio in African clawed frogs. The effects of perchlorate on metamorphosis are coincident with increases in thyroid gland hypertrophy and reductions in whole-body thyroid hormone concentrations. These effects are not restricted to laboratory studies; examination of tadpoles inhabiting perchlorate contaminated sites at Longhorn Army Ammunition Plant in East Texas suggests that similar effects are observed in tadpoles exposed to perchlorate under natural conditions (Carr et al., 2003).

The goal of this study was to examine naturally occurring frogs inhabiting the Lake Waco and Lake Belton watersheds for evidence of thyroid disruption. Our approach was twofold. First, we collected adult and larval frogs from reference and contaminated sites in and around McGregor, Texas and preserved these specimens for subsequent histological assessment of thyroid function. Second, we collected water samples from reference and contaminated sites in the vicinity of McGregor Texas, and transported the water samples back to the laboratory. Larval frogs were raised in the water samples to determine if thyroid-disrupting agents, including perchlorate, were present. Our working

hypothesis was that frogs exposed to biologically active concentrations of perchlorate in the environment would exhibit evidence of thyroid disruption based on thyroid histopathology endpoints, and that samples of surface water bodies contaminated with perchlorate would cause changes in developmental rate in laboratory exposed tadpoles.

5.4.2.1 Thyroid Histology of Native Frogs

5.4.2.1.1 Methodology

Seven trips were made to sites (see **Table 5-46**) in the NWIRP area (7/19/01, 10/10/01, 11/09/01, 5/7/02, 7/23/02, 4/21/03, and 6/16/03). GPS coordinates (UTM) were recorded for each using a Garmin GPS III Plus receiver. Pictures were taken of each site with a Nikon COOLPIX 995 digital camera. Frogs and tadpoles were collected in dip nets along the bank or in shallow water and immediately euthanized by immersion in MS-222 (3-aminobenzoic acid ethyl ester and NaHCO₃, each mixed in distilled water at 3 g/L). The animals were then stored in 10% neutral-buffered formalin (NBF). Sampling of most sites took approximately 1 hour and was conducted by 1-2 people. Whenever possible each site was visited in the morning and later in the afternoon. All animals were identified to species and measured for snout-vent length, hindlimb length, total length, and weight. Sex was determined by direct visual inspection of the gonads. Tadpoles were staged (Gosner, 1960), weighed, and measured for snout-vent length, hindlimb length, total length, and tail length and height. Water samples for perchlorate analysis were collected in 20 mL glass scintillations vials and placed in ice chests. See **Table 5-46** and **Figure 5-170** for a list of collection sites.

**Table 5-46
Field Sites Visited for the Amphibian Work**

Site ID	GPS (UTM)	Site Description	Water Collected for Perchlorate Analysis
NBSB (T14)	14R 0652873E 3476602N	South Bosque, south of water treatment plant, 3.3 miles south of Hwy 84	Y
HC84E (T34)	14R 0659507E 3483449N	Harris Creek at Highway 84 near the Executive Airport	Y
HCOOE (T17)	14R 0649179E 3478959N	Harris Creek east on Old Oglesby Rd 0.9 mi south of Hwy 84.	Y
HCGHA	14R 0649006E 3479236N	Harris Creek on Gladys Hollan's land, NE of old barn	Y
CROOR (T18)	14R 0649223E 3479242N	Spring on Old Oglesby Road, 0.2 mile south of Hwy 84	Y
ONCRN (T24)	14R 0645519E 3472070N	Onion Creek North of Hwy 107	Y
OLLO (T35)	14R 0664172E 3484662N	South Bosque at Highway 84 (Old Lorena Road)	Y
SBIT (T33)	14R 0655016E 3476016N	South Bosque at Indian Trail	Y
SBSBE	14R 0657527E 3477420N	South Bosque on unknown road	Y
MNSP (T28)	14R 0645313E 3465379N	Leon River (at Mother Neff Park)	Y
MGRB (T29)	14R 0650101E 3473922N	Creek on McGruffey RD	Y
MCGR (T13)	14R 0651011E 3480195N	Harris Creek at Highway 317	Y
PC317 (T3)	14R 0650195E 3483242N	Pecan Creek at Hwy 317	Y
SBSBL	14R 0654534E 3475300N	South Bosque at McGregor South Loop	Y
SOBO (T16)	14R 0653811E 3473890N	South Bosque at Highway 317, 4 miles south of Hwy 84	Y
SC107 (T23)	14R 0642978E 3471315N	Station Creek at Hwy 107	Y
SCOOR (T6)	14R 0642334E 3477056N	Station Creek at Oglesby road	Y
UN317 (T15)	14R 0653656E 3474994N	S Creek at Hwy 317	Y
SOBOW	14R 0647801E 3472718N	South Bosque on Hwy 2671-west side of road	Y
UB2671	14R 0646966E 3469992N	Unknown creek on Hwy 2671 0.75 mi south of McClennen Rd	Y
UCGHA	14R 0648944E 3479174N	Water from pipe draining into Harris Creek	Y

Follicle Cell Hypertrophy

- 0 No hypertrophy – follicles lined by squamous to cuboidal epithelium.
- 1 Follicles lined by tall cuboidal to columnar epithelium; cytoplasm; nucleus ratio increased.
- 2 Follicular epithelial cells distinctly larger than normal; follicular lumen severely decreased or obliterated.

Follicle Cell Hyperplasia

- 0 No hyperplasia – follicles lined by a single layer of normal appearing squamous to short cuboidal epithelium.
- 1 Two or more follicles exhibiting stratification of follicular epithelium, usually 2-3 cells thick, protruding into lumen (NOTE: follicles exhibiting stratification near gland periphery are not counted).
- 2 Greater number of affected follicles exhibiting stratification of follicular epithelium, usually more than 3 layers thick, protruding into lumen; area of hyperplasia may also have microfollicular formation within them.

Data were analyzed using either parametric or nonparametric statistics. Data were tested for homogeneity of variance using Bartlett's test. If the assumptions of parametric statistics were met, then Student's two-tailed t-test or ANOVA followed by the Tukey-Kramer multiple comparison test were used. If the assumptions of parametric tests were not met, then the Mann-Whitney nonparametric t-test or the Kruskal-Wallis (KW) ANOVA by ranks followed by Dunn's multiple comparisons test were used. All statistical analyses were performed using InStat (v. 2.05a, GraphPad Software, San Diego, CA) or SPSS (v. 11, SPSS Inc., Chicago, IL).

5.4.2.1.2 Data

The species and numbers of adult and larval frogs collected are reported in **Table 5-47**. By far the most abundant collected species was Blanchard's cricket frog, *Acris crepitans Blanchardi*. Adult *A. crepitans* were collected routinely throughout the study period at several sites, whereas larval *A. crepitans* were collected at fewer sites and only on the last trip in the spring of 2003. Because adult *A. crepitans* were collected at reference sites, as well as sites known to have measurable perchlorate in surface waters, we used these animals for the thyroid histopathological analysis. Morphological parameters and sex ratios for the adult *Acris crepitans* are reported below in **Table 5-48**. In general, male and females were equally represented at each site, and there was no evidence at the gross morphological level of intersexual gonads (Reeder et al., 1998; Carr et al., 2003).

Table 5-47
Adult and Larval Amphibians Collected by Species Between June 2001 and June 2003 in the Lake Waco and Lake Belton Watersheds

Site	Species							
	<i>A. crepitans</i>		<i>R. catesbeiana</i>		<i>R. blairi</i>		<i>Bufo valliceps</i> (adult only)	<i>Hyla versicolor</i> or <i>H. chrysoscelis</i> (larvae only)
	adults	larvae	adults	larvae	adults	larvae		
HC84E	2	10		17				
HCGHA	1							
MCGR	8							
NBSB	13							
NBSBR	5							
OLLO	39				2		3	3
ONCRN					2			
PC317	4	48		14				
SBSBE	7			2				
SBSBL	8							
SC107	2				1			
SCOOR								
SOBO	52	124		1	3			
SOBOW								
UA267				28				
UB267					10			
UN317	1						1	
Total	142	182		62	18		4	3

Table 5-48
Mean (\pm Standard Error) Snout-Vent Length (mm), Hindlimb Length (mm), Body Weight (g), and Sex Ratios in Field-Caught Adult Cricket Frogs (*Acris crepitans blanchardi*)

Site	N	SVL ^a	HLL ^b	Body Weight	Sex Ratio (M/F)	Perchlorate ($\mu\text{g/L}$) ^c
HC84E	2	21.5 \pm 6.50	32.0 \pm 9.00	0.76 \pm 0.43	0/2	0-4
HCGHA	1	23.0	39.0	1.03	1/0	0
MCGR	8	17.3 \pm 1.15	30.25 \pm 2.33	0.56 \pm 0.10	3/3	0-16
NBSB	18	25.0 \pm 2.08	40.3 \pm 2.78	1.99 \pm 0.51	6/12	0-232
OLLO	29	23.0 \pm 0.55	39.9 \pm 1.06	2.07 \pm 0.28	18/11	0-7
PC317	3	24.7 \pm 1.76	42.0 \pm 4.16	1.24 \pm 0.45	2/1	0
SBSBE	6	21.2 \pm 1.72	42.0 \pm 2.63	1.12 \pm 0.33	1/5	0-2.25
SBSBL	4	21.9 \pm 3.2	36.75 \pm 4.77	1.27 \pm 0.51	1/3	0-3.3
SC107	2	22.0 \pm 5.00	34.0 \pm 2.89	0.96 \pm 0.45	1/1	0-149
SOBO	39	22.7 \pm 0.61	38.9 \pm 1.08	1.24 \pm 0.09	14/20	0-31.3
UN317	1	20	34.0	0.9095	0/1	62-540

^aSVL, snout-vent length

^bHLL, Hindlimb length

^cRange of values measured between March 2001 and June 2003

Average perchlorate concentrations at the study sites used for thyroid histopathological analyses are shown in **Table 5-49**. The perchlorate concentrations were averaged from individual measurements performed between March 2001 and June 2003. Average perchlorate concentrations in surface water were statistically greater at site NBSB than all other sites. Average perchlorate concentrations at site MCGR were greater than SOBO and OLLO. The rank order for perchlorate concentrations at the study sites was NBSB > MCGR > SOBO > OLLO > SBSBL > SBSBE. There was no evidence of colloid depletion or follicle cell hyperplasia in any of the 86 animals studied. There was evidence for moderate follicle cell hypertrophy in animals from NBSB and MCGR, the two sites with the greatest measured perchlorate concentrations. Follicle cell hypertrophy was statistically greater in NBSB animals than animals from all other sites (**Figure 5-171**). Follicle cell hypertrophy was greater in animals from MCGR than in animals from OLLO and SOBO. The rank order for follicle cell hypertrophy based on collection sites was NBSB > MCGR > SBSBL > OLLO > SOBO > SBSBE. These findings suggested an association between follicle cell hypertrophy and average perchlorate concentrations at the study site. Hypertrophy data were plotted against mean perchlorate concentration and analyzed by Spearman's rank correlation. There was a significant positive correlation ($P < 0.0001$) between hypertrophy score and average perchlorate concentration in surface water, although the relationship explained only 36% of the variation in thyroid hypertrophy score amongst the animals studied (**Figure 5-172**).

Table 5-49
Mean (\pm Standard Error) Perchlorate Concentrations (ng Perchlorate/mL) in
Surface Water and Thyroid Histopathology Scores in Adult *Acris crepitans*
Collected in Lake Waco and Lake Belton Watershed

Site	Perchlorate	Colloid Depletion	Hypertrophy	Hyperplasia
SBSBE	0.95 \pm 0.39 ^{a,b} (n=7)	0.00 \pm 0.00 (5)	0.10 \pm 0.07 ^{a,b} (5)	0.00 \pm 0.00 (5)
SOBO	1.40 \pm 0.86 ^a (n=40)	0.00 \pm 0.00 (32)	0.15 \pm 0.05 ^a (32)	0.00 \pm 0.00 (32)
OLLO	1.20 \pm 0.43 ^a (n=28)	0.00 \pm 0.00 (25)	0.22 \pm 0.07 ^a (25)	0.00 \pm 0.00 (25)
SBSBL	1.01 \pm 0.55 ^{a,b} (n=7)	0.00 \pm 0.00 (3)	0.28 \pm 0.25 ^{a,b} (3)	0.00 \pm 0.00 (3)
MCGR	5.95 \pm 0.93 ^b (n=32)	0.00 \pm 0.00 (7)	0.65 \pm 0.13 ^b (7)	0.00 \pm 0.00 (7)
NBSB	25.9 \pm 8.32 ^c (n=37)	0.00 \pm 0.00 (14)	0.71 \pm 0.05 ^c (14)	0.00 \pm 0.00 (14)

Values with different superscripts are significantly different based on Kruskal-Wallis nonparametric ANOVA ($p < 0.05$).

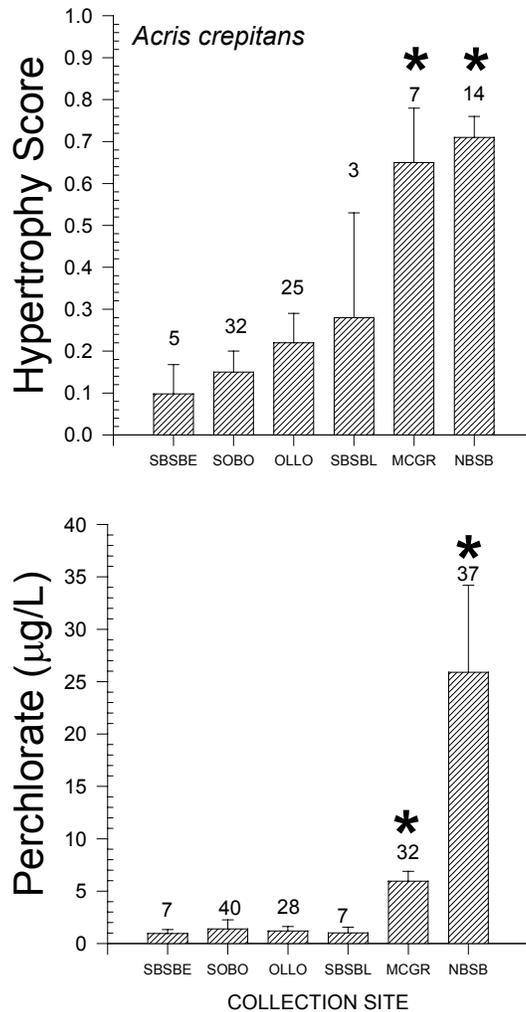


Figure 5-171
Thyroid Follicle Cell Hypertrophy Score (Upper Panel) and Average Perchlorate
Concentration at Sites Where Adult *Acris crepitans* Were Collected

Bares are the mean + S.E.M. Numbers above the bars indicate sample size.

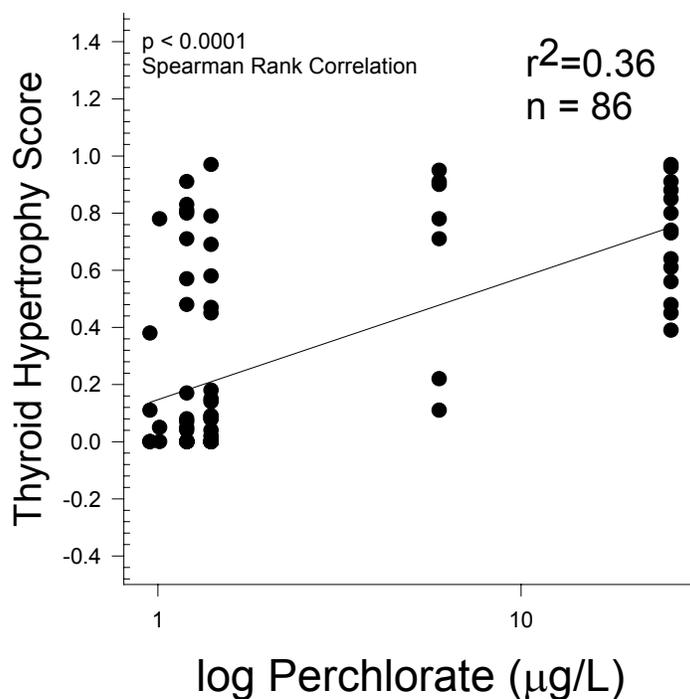


Figure 5-172
Thyroid Hypertrophy Score Plotted Against the Log of Mean Perchlorate Concentration for Sites at Which *Acris crepitans* Adults Were Collected

Each point represents the thyroid hypertrophy score for a single animal

Developmental stage and morphological characteristics of larval *Acris crepitans* collected sites in the Lake Waco/Lake Belton watersheds are shown in **Table 5-50**. Tadpoles at site PC317 were at a significantly earlier developmental stage, and were significantly smaller based upon snout-vent length (SVL), total length, and body weight, than tadpoles collected at HC84E or SOBO. None of the sites had appreciable perchlorate contamination. Perchlorate ranging from 3 to 4 µg/L was detected at HC84E in only 2 of 19 samples. Perchlorate was not detected in three samples collected from PC317 at the time of animal collection. Perchlorate was detected in 5 of 40 samples from SOBO ranging from 1.35 µg perchlorate/L to 31 µg perchlorate/L. Although hindlimb length, a sensitive indicator of perchlorate exposure under laboratory conditions, was smaller in animals collected from PC317, snout-vent length also was smaller in these animals. Under laboratory conditions perchlorate exposure specially affects hindlimb growth but does not influence overall somatic growth as gauged by snout-vent length (SVL).

Table 5-50
Mean (\pm Standard Error) Gosner Stage, Snout-Vent Length (SVL), Total Length, Hindlimb Length (HLL), Tail Length, Tail Height, and Body Weight in Larval *Acris crepitans* Collected in the Lake Waco and Lake Belton Watersheds

Site	N	Gosner Stage	SVL (mm)	Total Length (mm)	HLL (mm)	Tail Length (mm)	Tail Height (mm)	Body Weight (g)
HC84E	10	34.0 \pm 1.52 ^{a,b}	13.2 \pm 1.11 ^a	31.7 \pm 2.80 ^{a,b}	4.14 \pm 0.93 ^{a,b}	18.7 \pm 1.89 ^a	8.20 \pm 0.51 ^a	0.49 \pm 0.08 ^a
PC317	49	31.6 \pm 0.54 ^b	10.2 \pm 0.26 ^b	26.6 \pm 0.82 ^b	1.65 \pm 0.17 ^b	16.4 \pm 0.72 ^a	6.98 \pm 0.17 ^a	0.28 \pm 0.02 ^b
SOBO	124	33.0 \pm 0.37 ^a	11.2 \pm 0.22 ^a	30.0 \pm 0.81 ^a	4.57 \pm 0.50 ^a	18.9 \pm 0.60 ^a	7.21 \pm 0.17 ^a	0.46 \pm 0.02 ^a

^aCalculated as the number dead at completion of the experiment divided by the number of animals placed in the tank at the start of the experiment.

^bNumber reaching forelimb emergence (both forelimbs) divided by the number of animals placed in the tank at the start of the experiment.

^cNumber completing tail resorption divided by the number of animals placed in the tank at the start of the experiment.

5.4.2.1.3 Discussion

In the laboratory, perchlorate exposure throughout larval development results in thyroid colloid depletion, follicle cell hypertrophy, and follicle cell hyperplasia (Goleman et al., 2003; Hu et al., 2003). Full larval period exposures to concentration as low as 59 μ g perchlorate/L result in follicle cell hypertrophy. Furthermore, tadpoles (*Pseudacris triseriata*) exposed to greater than 9 ppm perchlorate in native ponds demonstrate colloid depletion and follicle cell hypertrophy. Our data indicate that frogs collected from NBSB and MGRB showed evidence of follicle cell hypertrophy but not colloid depletion of follicle cell hyperplasia. These findings are of interest because both NBSB and MGRB contained the greatest mean perchlorate concentrations of all of the sites studied. Qualitative histological analysis revealed that the follicle cell epithelium of the frogs collected from NBSB did not exhibit the gross alterations in size characteristic of exposure of *Xenopus laevis* tadpoles to mg/L concentrations of perchlorate under laboratory conditions. Furthermore, linear regression analysis of all of the hypertrophy scores against mean perchlorate concentration revealed that mean perchlorate concentrations accounted for only 36% of the variation in hypertrophy score, suggesting that other factors may also be involved. Dioxins and numerous other synthetic contaminants can influence thyroid function (Howdeshell et al., 2002), and the possibility that other contaminants may be involved has not been addressed.

Laboratory studies indicate that ammonium perchlorate delays metamorphosis and reduces hindlimb growth, a thyroid hormone dependent process in larval amphibians. Although tadpoles could not be collected at all of the study sites, significant numbers of tadpoles were collected at sites HC84E, PC317, and SOBO. Based on gross morphological measurements, including hindlimb length, there was no evidence that tadpoles from any of these sites were exposed to enough perchlorate to alter thyroid-hormone dependent features of metamorphosis.

One point that should be made is that comparing data from field collected specimens to data from numerous laboratory studies on perchlorate-inhibition of metamorphosis can be problematic, as laboratory studies generally employ a long and constant exposure period (in our lab studies a 70-day exposure) and data from field sites around Lake Waco indicate that perchlorate in surface waters varies considerably from month to month and year to year. For example, NBSB consistently had some of the greater perchlorate concentrations reported during the study period. Of 37 water samples from this site, there were several samples with no detectable perchlorate and many with perchlorate concentrations less than 10 $\mu\text{g/L}$ (**Figure 5-173**). Levels in October of 2001 were greater than 200 $\mu\text{g/L}$ however. Thus, developing frogs inhabiting the most contaminated sites in this study may have been exposed to considerably varying perchlorate concentrations and periods of no perchlorate exposure at all. This is significant as perchlorate effects on metamorphosis are reversible (Goleman et al., 2002b).

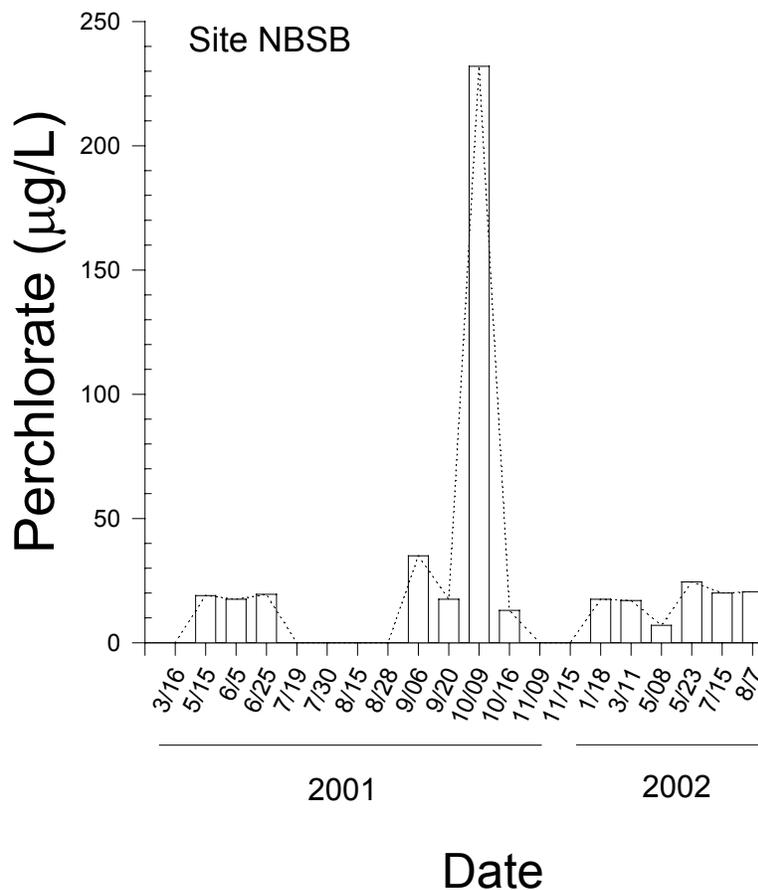


Figure 5-173
Perchlorate Concentrations Measured in Surface Water from NBSB Plotted Against Collection Date

5.4.2.2 *Developmental Assays Using Xenopus laevis, the African Clawed Frog*

5.4.2.2.1 Methodology

Sexually mature male and sexually mature oocyte positive HCG tested female *Xenopus laevis* were purchased from Xenopus Express and Nasco. Frogs were maintained in dechlorinated tap water in flow-through 160 L tanks at 19 °C at a density of 20 frogs per tank on a 12L:12D light regimen. Frogs were fed frog brittle (Nasco, Ft. Atkinson, WI, USA) three times weekly. Ammonia, pH, and specific conductance of the tank water were monitored every 7 days. Dissolved oxygen was monitored every 2 days, and water temperature monitored once daily. A YSI® model 85 meter (Yellow Springs, OH, USA) was used to monitor water temperature, percent saturation with dissolved oxygen, specific conductivity, and salinity for each tank. Free ammonium ion concentration and pH of the water in each tank were determined with a Hach® spectrophotometer model DR/2000 (Loveland, CO, USA) and an Oakton® pH meter (Gresham, OR, USA), respectively.

Breeding pairs were allowed to acclimate in 40-L glass aquaria containing 18 L FETAX medium (Dawson and Bantle, 1987) for 7 days prior to breeding. Naturally fertilized eggs were obtained from 5-8 pairs of adults as previously described (Goleman et al., 2002). Viable embryos were identified by visual observation with a binocular dissecting microscope. Embryos and tadpoles up to 5 days old were held in 9 L of FETAX medium in 21-L glass tanks acclimated to 22 °C (± 2 °C) on a 12L:12D regimen. Five-day old larvae were transferred to 45-L glass tanks containing 18 L FETAX or test solution. Tadpoles were fed 0.4 g of powered frog brittle (Nasco) every 48 hours. Tanks were checked daily for depletion of food and additional food supplied as necessary. All procedures involving *X. laevis* were approved by the Texas Tech Animal Care and Use Committee.

Water samples for developmental exposure of *X. laevis* tadpoles were collected in 4-L amber glass jugs. Eight-liters of water were collected from each of four or more sites. Water was collected in the middle of the stream or pond approximately 0.3 m off the bottom (in some cases the water was so shallow this was not possible). The water was kept as cool as possible in the field. Upon arrival in Lubbock the samples were stored at 4 °C. See **Table 5-46** for sites. Water quality measurements were recorded for temperature, dissolved oxygen, conductivity, salinity, and pH using a YSI® model 85 meter (Yellow Springs, OH, USA) and an Oakton® pH meter (Gresham, OR, USA).

Initially the experiment was performed using NF stage 55 tadpoles (Nieuwkoop and Faber, 1994). Eight liters of water were collected from each of four sites on two trips (OLLO, SBIT, SOBO, and NBSB) and from five sites on the third trip (OLLO, SBIT, SOBO, MGCR, and SC107). One site (NBSB) was dry on the last trip. Samples from each site were analyzed for ClO₄ ; samples were also collected from each beaker before the exposure and tested for ClO₄. The larvae were allowed to develop to NF stage 55 (Nieuwkoop and Faber, 1994) and snout-vent length (SVL), and tail length and height were recorded. Thirteen 2-L glass beakers containing 1-L of field water were setup. Six 2-L glass beakers containing 1-L of FETAX were setup as controls. Twenty tadpoles

were placed in each beaker. A 50% water change was made every 2 days and 0.5-L of site water was added back. Water was maintained at $22\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ with a 12L:12D regimen. Tadpoles were exposed until they reached stage NF 66. Mortality, number showing deformities, number showing abnormal swimming behavior, and number metamorphosed animals were recorded daily. Dead tadpoles were removed daily and preserved in 10% neutral-buffered formalin (NBF). At the end of the exposure each animal was staged (Nieuwkoop and Faber, 1994), and SVL, tail length and height, hindlimb length, total length, and weight were recorded. Every 7 days ammonia, pH, and specific conductance, and salinity were recorded. Water temperature was monitored daily. A YSI® model 85 meter (Yellow Springs, OH, USA) and an Oakton® pH meter (Gresham, OR, USA) were used. Free ammonium ion levels were determined with a Hach® spectrophotometer model DR/2000 (Loveland, CO, USA). Dissolved oxygen was measured for tadpoles every 2 days with the YSI® model 85 meter.

The EDSTAC Tier I frog metamorphosis has been criticized for lacking sensitivity, as the NF stage 60 animals that are used for this procedure already exhibit elevated blood levels of thyroid hormones; as a result the assay may not sensitively detect alterations in thyroid hormone synthesis. Data of ours collected from studies unrelated to the present study indicated that even stage 55 tadpoles may not be sensitive to the disrupting effects of perchlorate. To ensure that our laboratory exposures utilized tadpoles that would be sensitive to the perchlorate, we repeated the exposure studies using NF stage 49 tadpoles. The thyroid gland is not yet fully developed in stage 49 tadpoles and theoretically they would respond maximally to any disruption of thyroid hormone synthesis. For this assay larvae were allowed to develop to NF stage 48-49. One hundred eighty larvae were placed in six 10-L glass tanks (thirty larvae per tank), each containing 4-L of FETAX or field water from Trip 7. Two days later this was repeated. Medium was changed every three days. Larvae were exposed to the medium in each tank for 28 days. Each tank was maintained at $21\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ with a 12L:12D regimen. Every 7 days, water temperature, ammonia, pH, specific conductance, and salinity were recorded. A YSI® model 585 meter (Yellow Springs, OH, USA) was used. Free ammonium ion levels were determined with a Hach® spectrophotometer model DR/2000 (Loveland, CO, USA). Water temperature was also monitored daily by use of surrogate tanks. Before placement in the test solutions larvae were staged (Nieuwkoop and Faber, 1967) and snout-vent length and tail length were recorded. Each day the number of dead tadpoles, number showing deformities, and number displaying abnormal swimming behavior were recorded. At the end of the exposure (Day 28) the NF stage, snout-vent length, tail length and height, hindlimb length, and total length were recorded for each tadpole. After 28 days of exposure the tadpoles were euthanized by immersion in MS-222, and placed in Bouin's fixative. Water samples were taken from each carboy and analyzed for perchlorate.

Data were analyzed using either parametric or nonparametric statistics. Data were tested for homogeneity of variance using Bartlett's test. If the assumptions of parametric statistics were met, then Student's two-tailed t-test or ANOVA followed by the Tukey-Kramer multiple comparison test were used. If the assumptions of parametric tests were not met, then the Mann-Whitney nonparametric t-test or the Kruskal-Wallis (KW) ANOVA by ranks followed by Dunn's multiple comparisons test were used. All

statistical analyses were performed using InStat (v. 2.05a, GraphPad Software, San Diego, CA) or SPSS (v. 11, SPSS Inc., Chicago, IL).

5.4.2.2.2 Data

Surface water samples were collected from a number of sites in the Lake Waco/Lake Belton watersheds (**Table 5-51**). The number of experimental replicates and total number of tadpoles surviving for the 38-39 day exposure are also shown in **Table 5-52**. The majority of animals completed metamorphosis (NF stage 66) in all treatments (**Table 5-53**), including in the NBSB treatment which had the greatest perchlorate concentrations prior to testing. Mortality was low (3%) in the control FETAX medium exposure. All of the animals exposed to water from the SBIT4 site died within a day or two of exposure in all three replicates. Mortality also was high (60%) in the MGRB and SC107 sites. There were no obvious signs of edema in any of the exposed animals, although ten percent of the animals exposed to MGRB water (the same water that produced 60% mortality) exhibited abnormal swimming. Hindlimb growth (a sensitive indicator of thyroid disruption in tadpoles) did not differ amongst animals from any of the treatments relative to controls.

Table 5-51
Site Collection Data for the Frog Metamorphosis Assay

Site	Total N*	Replicates	GPS (UTM)	Location	Collection Date	Perchlorate (µg/L)**
CONT	97	5	NA	NA	NA	FETAX ^a
MGRB	8	1	650101E 3473922N	Unidentified Tributary at bridge on McGruffy Rd.	11/01	0
NBSB	28	2	652853E 3476589N	Unnamed Tributary near Wastewater Treatment Plant	8/01-10/01	0-232
OLLO	55	3	664177E 3484767N	South Bosque at Highway 84	8/01-11/01	0-3.61
SBIT	39	2	655034E 3476011N	South Bosque at Indian Trail	8/01-10/01	0-9.56
SBIT 4	0	3	655034E 3476011N	South Bosque at Indian Trail	11/01	5.82-8.76
SC107	9	1	642988E 3471304N	Station Creek at Highway 107 (T23)	11/01	10.27-22.07
SOBO	56	3	653810E 3473908N	South Branch of South Bosque at Highway 317 (T16)	8/01-11/01	0-31.3

*Total surviving, 20 animals per replicate. All animals in SBIT-4 died before the end of the experiment.

** Measured in the surface water samples used for testing.

^aNo perchlorate detected in FETAX medium.

Table 5-52
Mean ± Standard Error Mortality, Forelimb Emergence, Tail Resorption, Edema, Bent Tails, and Abnormal Swimming in *Xenopus laevis* Tadpoles Exposed to Surface Water for 38-39 Days Beginning at Stage 55

Site	Mortality ^a (%)	Forelimb Emergence ^b (%)	Tail Resorption ^c (%)	Edema (%)	Bent Tails (%)	Abnormal Swimming (%)
CONT	3.00 ± 1.23	97.0 ± 3.00	89.0 ± 4.58	0.00 ± 0.00	0.00 ± 0.00	2.00 ± 1.23
MGRB	60.0	35.0	30.0	0.00 ± 0.00	0.00 ± 0.00	10.0 ± 0.00
NBSB	30.0 ± 10.0	80.0 ± 15.0	60.0 ± 20.0	0.00 ± 0.00	0.00 ± 0.00	15.0 ± 5.00
OLLO	8.33 ± 4.41	98.3 ± 1.67	90.0 ± 5.00	0.00 ± 0.00	0.00 ± 0.00	0.00
SBIT	2.50 ± 2.50	100	97.5 ± 2.50	0.00 ± 0.00	0.00 ± 0.00	0.00
SBIT 4	100	0.00	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00
SC107	55.0	45.0	45.0	0.00 ± 0.00	0.00 ± 0.00	0.00
SOBO	6.67 ± 3.33	96.7 ± 3.33	91.7 ± 4.41	0.00 ± 0.00	0.00 ± 0.00	0.00

^aCalculated as the number dead at completion of the experiment divided by the number of animals placed in the tank at the start of the experiment.

^bNumber reaching forelimb emergence (both forelimbs) divided by the number of animals placed in the tank at the start of the experiment.

^cNumber completing tail resorption divided by the number of animals placed in the tank at the start of the experiment.

Table 5-53
Mean ± Standard Error Developmental Stage, Snout-Vent Length (SVL), Hindlimb Length, Total Length, Tail Length, Tail Height, Body Weight, and Sex Ratio in *Xenopus laevis* Tadpoles Exposed to Surface Water for 38-39 Days Beginning at Stage 55

Site	Nieuwkoop-Faber Stage	SVL (mm)	HLL (mm)	Total Length (mm)	Tail Length (mm)	Tail Height (mm)	Body Weight (gm)	Sex Ratio (M/F)
CONT	65.6 ± 0.18	16.7 ± 0.17	18.8 ± 0.42	17.8 ± 0.58	1.14 ± 0.58	0.21 ± 0.09	0.46 ± 0.01	50.0%
MGRB	64.5 ± 1.24	14.8 ± 0.43	15.0 ± 2.22	15.0 ± 2.23	4.75 ± 4.34	0.63 ± 0.63	0.38 ± 0.05	50.0%
NBSB	65.5 ± 0.32	16.8 ± 0.29	19.0 ± 0.81	18.2 ± 1.12	1.35 ± 1.09	0.29 ± 0.18	0.49 ± 0.02	80.0%
OLLO	65.8 ± 0.16	16.1 ± 0.26	18.5 ± 0.43	16.7 ± 0.67	0.64 ± 0.63	0.10 ± 0.09	0.42 ± 0.02	37.9%
SBIT	66.0 ± 0.03	16.8 ± 0.25	19.1 ± 0.34	16.8 ± 0.25	0.03 ± 0.03	0.03 ± 0.03	0.46 ± 0.02	55.6%
SBIT 4	NA	NA	NA	NA	NA	NA	NA	NA
SC107	66.0 ± 0.00	16.3 ± 0.45	19.4 ± 1.03	16.3 ± 0.45	0.00 ± 0.00	0.00 ± 0.00	0.48 ± 0.04	33.3%
SOBO	65.8 ± 0.16	16.2 ± 0.20	18.4 ± 0.46	16.8 ± 0.58	0.54 ± 0.54	0.09 ± 0.09	0.45 ± 0.02	51.9%

Recently, Opitz et al. (2002) have proposed an alternative to the flawed EDSTAC frog metamorphosis assay. In the XEMA assay (*Xenopus* metamorphosis assay), NF stage 49 tadpoles are used. In theory these animals would be more sensitive to the thyroid disrupting agents, as exposures would begin before NF stages 51-52, when the thyroid gland is fully formed. In the Fall of 2003 we performed some preliminary trials using the XEMA assay to test water samples collected from CROOR, MNBP, NBSB, PC317, and UN317. In these tests we spent a considerable amount of time making sure that NF stage 49 tadpoles only were used in the tests. Unfortunately we encountered very high mortality (greater

than 80%) in all of these trials. This mortality is unacceptably high, and occurred even in the controls (contrast this to mortality in the EDSTAC experiments which was 3%).

5.4.2.2.3 Discussion

As it was originally described, the EDSTAC Tier I frog metamorphosis assay (Fort and Stover, 1997; Federal Register, 1998) was designed to use NF stage 66 tadpoles in a 14- or 21-day exposure. The overriding problem with such an approach is that the thyroid-hormone dependent gene program that initiates metamorphosis is already well-under way in NF stage 60 tadpoles, and plasma thyroid hormones levels are near maximal in these animals (Leloup and Buscaglia, 1977). Thus, one would not predict that the frog metamorphosis assay as it is designed would be sensitive enough to detect chemicals that alter thyroid hormone synthesis. For this reason, the EDSTAC procedure was modified so that it began at NF stage 55 rather than NF stage 60. The data on metamorphosis as well as the measurement of perchlorate in the surface water samples tested together suggest that none of the samples had sufficient perchlorate concentrations to alter the rate of metamorphosis. Animals completed metamorphosis in all of the tested samples except for MCGR, which showed slightly slower metamorphosis, although a high percentage of the animals exposed to water from this site died before the end of the testing period. Water from one site SBIT4 killed all of the test animals in all three trials. Given that the minimal perchlorate concentration capable of fully blocking metamorphosis in *X. laevis* in the laboratory is 147 mg/L under constant 70-day exposure (Goleman et al., 2002a), it is likely that none of the sites studied would lead to a complete blockade in metamorphosis.

Data collected from the 28-day exposure using stage 49 larvae revealed high but delayed mortality in all treatments, most likely due to the handling required to accurately stage the NF stage 49 larvae. These handling effects resulted in none of the animals, including controls, developing normally during the 28-day exposure. The mortality was traced to the methods employed for selection of NF stage 49 animals. These larvae were held on ice-chilled FETAX medium to reduce movement so that they could be observed under the dissecting microscope to identify stage. At present there are no accepted and standardized guidelines for the 28-day partial life cycle test. A solution for future studies would be to use age-matched tadpoles whose stage on average was stage 49 for that post-hatching date.

5.4.2.3 Gonadal Effects on Native and Non-Native Frogs

5.4.2.3.1 Methodology

Sex ratios were determined by direct visual inspection of field-collected animals or *X. laevis* from the laboratory exposure studies as described previously (Goleman et al., 2002a). Previously fixed specimens were rinsed in deionized water and pinned to a dissecting dish of hardened paraffin wax placed under a binocular dissecting microscope with a high intensity illuminator as a light source. The abdominal cavity was opened and the intestines removed. The kidneys were located in the retroperitoneal region. Appearance of the gonads depended upon the age of the animal and the species, but in general the ovaries were long and lobular, with small areas of dark pigmentation visible.

Testes were shorter and lacked lobes, but varied in pigmentation based upon the species. Gonadal abnormalities were scored using the following criteria: Intersex gonads were categorized as: left/right intersex (a testis on one side and an ovary on the opposite side), rostral/caudal intersex (testicular characteristics rostrally and ovarian characteristics caudally or vice versa), or mixed sex (mixed testicular/ovarian tissue). For purposes of analysis all three subcategories were combined and referred to as intersex.

After assessment of gonadal morphology, the dorsal wall of the abdominal cavity with kidneys and gonads attached was processed for routine paraffin embedding. Gonads were sectioned at 10 μM , stained with hematoxylin and eosin, and gonadal morphology examined using a light microscope.

Sex ratio and intersex were reported as incidence per treatment and were transformed (arcsine of the square root) before ANOVA in the case on intersex data or analyzed by Chi-square for sex ratio data.

5.4.2.3.2 Data

Phenotypic sex of field caught *Acris crepitans* was determined by gross morphological assessment of gonadal tissue and confirmation of a subset of animals by histology. There were no unambiguous sex (i.e. intersex) animals noted based on gross morphological assessment. The ratio of females to males was approximately 2:1 at NBSB, the site where the greatest perchlorate concentrations were observed. However, X^2 analysis revealed that this distribution was not significant, probably due to the rather small sample size.

In the frog metamorphosis assay, the sex ratio was distributed equally among 97 animals in 5 replicates, although exposures in this study began at stage 55, past the window of sex differentiation. In other words, neither the EDSTAC frog metamorphosis assay or our modification of the assay is designed to detect changes in phenotypic sex ratio because exposures begin after the critical window for gonadal sex differentiation, which is NF stage 44-50 in *Xenopus laevis* (Villalpando et al., 1990). This is not to say that the exposures could not affect gonadal growth or development in the study, however gonadal size was not evaluated in the current study and there are no reports on the effects of perchlorate exposure on gonadal growth. The unusual sex ratio observed for NBSB (80% males) is most likely due to a high percentage of males in one test tank prior to the onset of exposures. There is no evidence that perchlorate or thyroid disruption in general causes masculinization in *Xenopus laevis* or any other frog species.

5.4.2.3.3 Discussion

There was no evidence of intersexuality in captured *A. crepitans* from any of the field sites and no obvious trends between incidence of females and perchlorate in surface water. In *X. laevis*, exposure to 59 μg perchlorate/L for 70 days alters the sex ratio toward more females (Goleman et al., 2002b). In the current study there were no sites with perchlorate consistently detectable at greater than 50 μg perchlorate/L with the exception of Station Creek SC107 and UN317. There was no evidence in any of our trips that either of these sites was inhabited by amphibians. Moreover, it is unclear whether thyroid disruption would alter sex differentiation in this species as it does in *Xenopus laevis*.