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Thyroid status in juvenile alligators (*Alligator mississippiensis*) from contaminated and reference sites on Lake Okeechobee, Florida, USA

Elizabeth A. Hewitt [a], D. Andrew Crain [a,*], Mark P. Gunderson [b], Louis J. Guillette Jr [b]

[a] *Department of Biology, Maryville College, 502 Lamar Alexander Parkway, Maryville, TN 37804, USA*
[b] *Department of Zoology, 223 Bartram Hall, University of Florida, Gainesville, FL 32611, USA*

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Abstract

Exposure to environmental contaminants has been shown to alter normal thyroid function in various wildlife species, including the American alligator (*Alligator mississippiensis*). Abnormalities in circulating levels of the thyroid hormone thyroxine (T_4) have been reported in juvenile alligators from several contaminated lakes in Florida. To further elucidate these functional thyroid abnormalities, this study examines the structure of thyroids and circulating T_4 concentrations from juvenile alligators collected from three sites of varying contamination on Lake Okeechobee, Florida. The following variables were used to characterize thyroid morphology: epithelial cell height, width and area, percent colloid, and follicle area. These variables were compared among study sites and between genders. No difference was detected in epithelial cell height, epithelial cell area, or follicle area among the sites, whereas significant differences in epithelial cell width ($p = 0.02$) and percent colloid ($p = 0.008$) were found. Animals from the most contaminated site (Belle Glade) had significantly greater epithelial cell widths and significantly less colloid present in their follicles compared to animals from the reference site (West). Gender did not have a significant interaction with site for any variable measured. Thyroxine (T_4) concentrations were elevated in the intermediately contaminated site (Conservation Area 3A) compared to the other sites ($p < 0.0001$). It is proposed that the disruptions seen in Lake Okeechobee alligators are due to disruptions at both the thyroid and extra-thyroidal tissues.

Keywords: Alligator; Endocrine disruption; Contaminants; Thyroid gland, thyroxine

1. Introduction

The thyroid is the endocrine gland responsible for the synthesis and secretion of the hormones thyroxine (T_4) and triiodothyronine (T_3). Thyroid hormones are essential for the regulation of several major functions in all vertebrates, including metabolism and growth and development of body systems; other specific functions include regulating metamorphosis in amphibians and influencing reproduction (Schmidt-Nielsen, 1990; Dellovade et al., 1995). In light of the various roles of thyroid hormones, the consequences of thyroid disruption can be severe. Embryonic effects of thyroid deficiency include limited development of skeletal, muscle, and central nervous systems (McNabb and King, 1993).

Several recent studies have noted altered thyroid activity as a result of exposure to environmental chemicals,

* Corresponding author. Tel.: +1-865-981-8238; fax: +1-865-981-8010.

E-mail address: crain@maryvillecollege.edu (D.A. Crain).

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specifically endocrine-disrupting contaminants (EDCs). Although some of the most profound effects of EDCs are elicited in the reproductive glands of the endocrine system, thyroid gland disruption was one of the first observed effects of these chemicals and has since been well documented (see reviews in Leatherland (1992, 2000)). Elevated circulating T_4 levels coupled with depressed T_3 levels are noted in freshwater catfish (*Clarias batrachus*) experimentally exposed to the pesticide endosulfan (an organochlorine), suggesting that this pesticide blocks extrathyroidal conversion of T_4 to T_3 (Sinha et al., 1991). Unlike endosulfan, carbaryl (a carbamate) caused a decrease in T_4 levels and an increase in T_3 levels in *C. batrachus* (Sinha et al., 1991). A similar correlation between contaminant exposure and thyroid dysfunction has been noted in rats. Extreme hypothyroidism in weanling male rats was provoked by administration of the PCB mixture Aroclor 1254 (Gray et al., 1993).

In addition to functional abnormalities, numerous studies have noted structural abnormalities in the thyroids of wildlife exposed to environmental contamination. The constituents of thyroid follicles (epithelial cells, colloid, and individual follicles themselves) are instrumental in observing structural effects of EDCs on the thyroid. Histologically, epithelial cell height is the most frequently used method of thyroid gland assessment, as it is considered to be roughly proportional to the degree of response to thyroid-stimulating hormone, TSH (Moccia et al., 1981). Also useful are epithelial cell width, colloid content, and follicle diameter. Normal thyroid morphology—smaller follicles with columnar epithelial cells and abundant colloid—may be altered with prolonged exposure to contamination. Large follicles with cuboidal or flattened epithelial cells and severely depleted colloid have been observed in herring gulls from the Great Lakes basin, an area exhibiting prevalent thyroid disorder in fish, avian, and mammal species (Moccia et al., 1986; Leatherland, 1994). These structural abnormalities noted in Great Lakes species are thought to have an environmental etiology (Moccia et al., 1981).

Recently, thyroidal abnormalities have been noted in American alligators (*Alligator mississippiensis*). This species, as for reptiles in general, is highly susceptible to endocrine disruption by contamination (Crain and Guillette, 1998). Reproductive abnormalities persisting in the juvenile alligator population of Lake Apopka (Florida), site of a major pesticide spill in 1980, include altered sex-steroid concentrations, altered gonadal morphology, and reduced phallus size. Juvenile alligators collected from Lake Okeechobee, Florida (a site of significant agricultural runoff) have similar, although less pronounced, abnormalities in sex-steroid concentrations (Crain et al., 1998). In addition to reproductive abnormalities, altered thyroid hormone concentrations have been observed in Lake Okeechobee juvenile alligators (Crain et al., 1998). Circulating levels of thyroxine were significantly higher in Okeechobee male alligators compared to control animals. The present study seeks to examine both thyroid structure and thyroid function in Okeechobee juvenile alligators in order to elucidate previously reported functional abnormalities in this population. It is hypothesized that thyroid morphology and thyroxine concentrations will differ in animals from contaminated and reference sites.

2. Materials and methods

2.1. Study sites

Lake Okeechobee (lat. 26°56′N, long. 80°49′W), is the largest lake in Florida and was chosen for this study based on its historical exposure to environmental contamination as well as the previously reported alterations in thyroid status of its alligators. In the South Florida Water Management District (SFWMD), which is responsible for water-resource management in South Florida, the total estimated annual usage of pesticides as of 1996 was 14 590 tons/year (Miles and Pfeuffer, 1997). Pesticides including insecticides, herbicides, and fungicides are used in this area primarily for agriculture (sugarcane, citrus, and vegetable crops). Other uses include golf course maintenance, domestic uses, and mosquito control (Miles and Pfeuffer, 1997).

Animals were collected from three sites on or near Lake Okeechobee: Belle Glade (May 11, 1999), West (May 10, 1999), and Conservation Area 3A North (May 12, 1999). Belle Glade is located in the Southeastern portion of the lake and is adjacent to major sugarcane agriculture. In fact, 100% of Florida's sugarcane industry is located on the Southern-most edge of Lake Okeechobee (see Fig. 1). The herbicides ametryn, atrazine, and 2,4-D are predominantly used on sugarcane (Miles and Pfeuffer, 1997). West, an area considered to be relatively pristine, is located in the western-most part of the lake, an area that has little agriculture (see Fig. 1). Conservation Area 3A is the largest of five Conservation Areas located South of Lake Okeechobee and North of the Everglades National Park (see Fig. 1). These Conservation Areas, created in the 1950s and 1960s for water-management purposes, are shallow wetlands enclosed by levees. Conservation Area 3A releases water into the major undisturbed part of the Everglades (Mattraw et al., 1988). Water inflow and outflow structures located throughout the Conservation Areas represent major links in the water conveyance system from Lake Okeechobee and the Everglades Agricultural Area to the Everglades National Park (Lutz, 1977; Mattraw et al., 1988). Animals were collected in the Northern part of area 3A near inflow structure S-8.

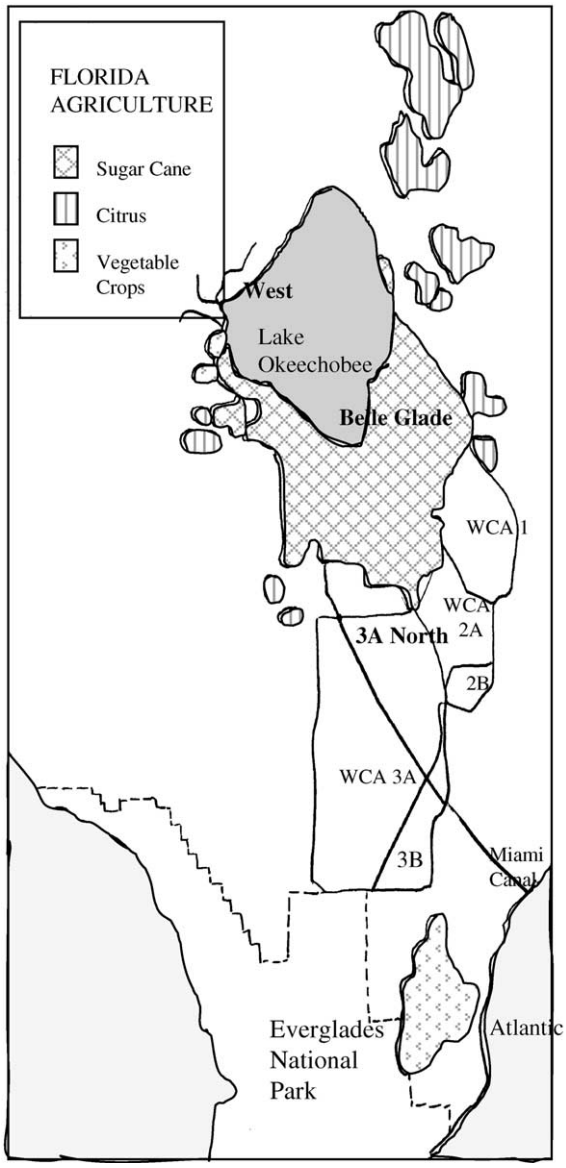

Fig. 1. A Map of South Central Florida. The three study sites (West, Belle Glade, and Conservation Area 3A North) are shown with their position relative to different agriculture practices (adapted from Miles and Pfeuffer (1997, p. 338)).

Pump station S-8 is adjacent to the Everglades Agricultural Area and is subject to extensive agricultural usage, primarily sugarcane (see Fig. 1).

Historically, the three sites have been differentially exposed to agricultural toxicants. In a pesticide residue study conducted from 1984 to 1988 within the SFWMD, chemicals (especially DDT metabolites) were consistently found in water and sediment samples obtained at pump station S-2 at the Belle Glade site; no compounds were found at detectable levels in water or sediment samples obtained from station FECSR78 at the West site; and four compounds were found at detectable levels in samples from pump station S-8 in Conservation Area 3A North (Pfeuffer, 1991; see Table 1). These data indicate that Belle Glade and surrounding areas have been sites of persistent agricultural runoff and environmental contamination, whereas detectable levels of chemicals in the West location have seldom been found. Conservation Area 3A North appears to be an area of moderate contamination.

2.2. Animal collection and sample processing

Juvenile alligators were hand-caught from an airboat over a period of three nights. Immediately upon capture, a 3 ml blood sample was collected in a vacutainer treated with sodium heparin. Samples were stored on ice for 6–9 h before centrifugation. Plasma was stored at −72 °C prior to radioimmunoassay for T_4, which was conducted as previously described (Crain et al., 1998). Briefly, 100 μl of unextracted plasma was incubated with T_4 antibody (1:2000 final concentration; Endocrine Sciences, Calabasas Hills, CA) and T_4 radiolabel (50 000 cpm/tube; New England Nuclear) in a 0.5 M borate buffer supplemented with 1% BSA, 1.25 mg/ml γ-globulin, and 2 mg/ml 8-alimino-1-naphthalene-sulfonic acid. Hundred μl of plasma stripped of T_4 was added to each standard tube. Tubes were incubated at 37 °C for 2 h, followed by room temperature incubation for 1.5 h. Bound-free separation was accomplished by adding 1.5 ml of 60% saturated ammonium sulfate to each tube, vortexing, and centrifuging at 1500 g for 30 min. The pellet was resuspended in a 9:11 mixture of saturated ammonium sulfate and assay buffer with 0.5% bovine serum albumin. After vortexing and centrifugation, the pellet was counted on a Beckman gamma counter.

After blood was collected from the alligators, weight, gender, and size (snout-vent length and total length) were determined for each animal. Approximately 10 animals (5 males and 5 females) were kept from each site. Animals were tagged and taken to the field station where they were euthanized by administering a lethal injection of sodium pentobarbital into the postcranial sinus. The thyroid gland was removed and immediately placed in Bouin's fixative. Once the fixative had permeated, the preserved tissues were transferred to 75% alcohol for clearing.

A histological analysis of the juvenile thyroids was performed in order to study the structure of the glands. The tissues remained in a solution of 75% alcohol prior to histological processing. Due to the large size of juvenile alligator thyroids, the tissues were cut in half using a scalpel. Tissues were embedded in paraffin wax, sectioned at 7 μm, and stained with a modified Harris'

Table 1
Environmental chemicals found at detectable levels in water and sediment samples on Lake Okeechobee and surrounding Conservation Areas (data compiled from Pfeuffer (1991))

Compound	Date	Belle Glade	Conservation Area 3A North	West
Zinc phosphide	June 1986	6 μg/l	3 μg/l	NS
Atrazine	May 1987	0.4 μg/l	0.3 μg/l	0
p,p′DDD	Feb. 1988	7.9 μg/kg	0	0
p,p′DDE	Oct. 1987	59.6 μg/kg	0	0
	Feb. 1988	10.0 μg/kg	0	0
	July 1988	28.0 μg/kg	0	0
Diazininon	Feb. 1986	1100 μg/kg	1100 μg/kg	0
Ametryn	July 1987	98.5 μg/kg	0	0
Malathion	Feb. 1986	3200 μg/kg	3300 μg/kg	NS

trichrome procedure as previously described (Presnell and Schreibman, 1997).

2.3. Histological analyses

In order to characterize the structure of the thyroid glands of juvenile alligators, several indices were measured including follicle height and width, epithelial cell height and width, and percent colloid. Ten follicles were randomly selected from each tissue. Only spherical follicles that were complete cross-sections were measured. All measurements were obtained using a Nikon Alphaphot-2 YS2 microscope with an ocular micrometer.

The width and height of a chosen follicle were measured at either 100× or 400× magnification depending on the size of the follicle. The diameter of each follicle was determined using the equation for calculating the diameter of an ellipse, $\pi/4(AB)$, where A and B represent height and width. For epithelial cell height, the cell with the greatest height was measured at a magnification of 400×. The same epithelial cell was used to obtain a measurement for epithelial cell width at 1000× magnification. The area of that cell was then determined using the equation for calculating the area of a rectangle, lw, where l and w represent height and width, respectively. The amount of colloid present in each follicle was quantitatively measured using digital image analysis. For the 10 follicles from each thyroid, a Nikon 990 digital camera captured the image and then the percent colloid was determined by image analysis software provided by the National Institutes of Health (NIH image).

2.4. Statistical analysis

For each of the variables, an analysis of variance (ANOVA) was conducted to determine if there were any differences among sites. First, the interaction of site and gender was analyzed to determine if gender was a significant variable. If not, gender was removed from the analysis and a single factor ANOVA was conducted to determine if the response variables differed among sites. In order to determine where the variation was found, the Tukey–Kramer post-hoc method was conducted for data found to be significantly different by the ANOVA test.

3. Results

In respect to thyroxine concentrations, there was no interaction between gender and site ($p = 0.90$). However T_4 was significantly different among sites ($p \leqq 0.0001$), with animals from Conservation Area having significantly higher T_4 concentrations compared to animals from both Belle Glade ($p = 0.0001$) and West (<0.0001). Belle Glade and West were not different from each other ($p = 0.64$).

The variables used to characterize thyroid morphology (epithelial cell height, width, and area, percent colloid, and follicle area) were first analyzed to determine if there was a significant interaction between gender and site. There was no such significant interaction for any of these morphological variables (epithelial height $p = 0.69$; epithelial width $p = 0.14$; epithelial area $p = 0.19$; percent colloid $p = 0.29$; follicle area $p = 0.29$). Thus, gender was removed from the analysis and morphological variables were compared among the three sites.

Table 2 presents mean values for the morphological variables and thyroxine concentrations in animals from the three study sites. There was no difference in mean epithelial cell height ($p = 0.13$), mean epithelial cell area ($p = 0.24$), or follicle area ($p = 0.46$) among the three sites. Significant differences were found in percent colloid ($p = 0.008$) and epithelial cell width ($p = 0.02$) among study sites. The Tukey–Kramer method revealed that Belle Glade animals, compared to animals from the reference site (West), had significantly less colloid present within their thyroid follicles and had significantly greater epithelial cell width. This gave the cells a more cuboidal or flattened appearance compared to the columnar shape of normal cells.

Table 2
Mean values (±1 SE) for measured variables in alligators from the study sites

	Belle Glade	Conservation Area	West	*p*-Value
Epithelial cell height (μm)	12.92 ± 1.08	15.42 ± 0.80	14.68 ± 0.97	0.129
Epithelial cell width (μm)	5.44 ± 0.27^{a}	4.86 ± 0.26	4.17 ± 0.39^{b}	0.0234
Epithelial cell area (μm^2)	66.11 ± 3.75	72.46 ± 3.00	60.56 ± 6.53	0.241
Percent colloid	80.36 ± 3.19^{a}	89.90 ± 2.61	93.39 ± 2.53^{b}	0.008
Follicle area (μm^2)	$8.41 \times 10^4 \pm 3.3 \times 10^4$	$4.72 \times 10^4 \pm 6.5 \times 10^3$	$5.40 \times 10^4 \pm 1.2 \times 10^4$	0.459
Thyroxine (ng/ml)	3.64 ± 0.32^{a}	7.32 ± 0.96^{b}	3.25 ± 0.24^{a}	<0.0001

[a,b] Values with different superscripts are significantly different at $p \leqslant 0.05$, as indicated by the Tukey–Kramer post hoc method.

4. Discussion

The results of this study indicate that both structural and functional differences in thyroid status exist among juvenile alligators collected from different sites on Lake Okeechobee. Structurally, epithelial cell width and percent colloid were found to be significantly different among the three study sites. Animals from the most contaminated site, Belle Glade, showed increased epithelial cell widths and decreased percent colloid compared to animals collected from the reference site, West. A relationship between gender and thyroid morphology could not be established in this study. Functionally, animals from the intermediately contaminated site (Everglade Conservation Area 3A) exhibited elevated thyroxine (T_4) concentrations compared to animals from both Belle Glade and West. Crain et al. (1998) found elevated T_4 concentrations in alligators taken from the North shore of Lake Okeechobee, similar to that found in animals from the Conservation Area 3A.

Both histological and hormonal data have been used to indicate thyroid endocrine disruption. Recently, it has been suggested that multiple indices are needed to accurately characterize such disruptions (Capen, 1998; O'Connor et al., 1999). Considering histological endpoints, epithelial cell width and percent colloid are useful measurements with which to assess thyroid function. Epithelial cell shape, which is indicative of thyroid activity, can be described by epithelial cell width. Columnar-shaped epithelial cells, characterized by small cell widths, are usually found in follicles that have been actively stimulated by TSH. An unstimulated follicle tends to have cuboidal-shaped epithelial cells, characterized by large cell widths (Leatherland, 1994). The amount of colloid present in a follicle is also an indicator of thyroid function, with less colloid indicating hormonal secretion from the gland (Moccia et al., 1981). Considering hormonal endpoints, T_4 concentration is often used to assess disruption, as it is the major circulating thyroid hormone (McNabb and King, 1993).

Recent studies of thyroid disruption have shown concurrent alterations in histological and hormonal parameters. Zhou et al. (1999) found that mummichogs (*Fundulus heteroclitus*) exposed to heavy metals and organic materials had larger thyroid follicles, greater epithelial cell heights, and elevated circulating T_4 concentrations. Similarly, rats exposed to low levels of acrylamide exhibit decreased colloid area with a corresponding increase in plasma T_4 (Khan et al., 1999). However, the current study found that animals from the most contaminated site (Belle Glade) had significantly reduced colloid but apparently normal thyroxine. Additionally, animals from the moderately contaminated site (Conservation Area) had normal histological parameters but significantly elevated plasma T_4. It is expected that as animals decrease their colloid (i.e., secrete thyroid hormones into the blood), the plasma T_4 will rise. However, this is obviously not the case in animals of both Belle Glade and Conservation Area, and there are two possibilities for the discrepancy between tissue and blood parameters.

First, there could be tissue-level thyroid disruption, but the discrepancy could be a factor of the one-time sampling event. The results of this study indicate that Belle Glade animals, compared to those from the reference site, have either slightly flattened or cuboidal-shaped epithelial cells coupled with a depleted source of colloid, suggesting that thyroid hormone secretion has occurred without concurrent thyroid hormone production (hormonogenesis). Similar abnormalities in colloid content have been noted in salmon and herring gulls from the Great Lakes (Moccia et al., 1981, 1986). Follicles largely depleted of colloid were common to both species; however, Moccia et al. found epithelial cells to be columnar in shape, unlike the cuboidal-shaped epithelial cells of alligators in this study. If thyroid-level disruption was occurring in the alligators, we would assume that the Belle Glade animals would have eventually had reduced T_4 (corresponding with their reduced colloid) and that previous to the sampling, Conservation

Area animals had reduced colloid. This is supported by a recent study that found distinct phases of thyroid activity (hormonogenesis and hormone release), with a lack of concurrent association between thyroid hormone and colloid appearance (Raine and Leatherland, 1999).

Second, there may be thyroid endocrine disruption both at the thyroid and at a site peripheral to the thyroid. Some xenobiotics have been show to disrupt thyroid economy by inducing hepatic microsomal enzymes (Capen, 1997). Elevating such hepatic enzymes would cause increased urinary excretion of thyroid hormones, and this would explain the results from the Belle Glade animals; whereas T_4 production histologically appears to be elevated (i.e., reduced colloid), the circulating T_4 concentration is normal. It has been suggested that decreased T_4 concentrations in male rats exposed to PCBs result from increased hepatic metabolism of T_4 by the liver (Gray et al., 1993). If organochlorines are elevating microsomal hepatic enzymes at the same time that T_4 production is elevated, we would expect to see reduced colloid coupled with "normal" T_4 levels. This hypothesis of thyroid disruption peripheral to the thyroid is supported by the abundance of organochlorine contaminants at the Belle Glade site; organochlorines are known to induce hepatic microsomal enzymes (Capen, 1997).

Of these two possibilities, we support the latter because a combination of both thyroid gland and hepatic disruption fully explains our data. Using West as our reference site, the intermediately contaminated Conservation Area shows elevated circulating T_4, suggesting an increase in hormone secretion. Table 1 shows that contaminants in animals from Conservation Area are similar to those from Belle Glade with the exception of elevated organochlorines in the Belle Glade alligators. Therefore, animals from Belle Glade appear to have both elevated hormonal secretion and urinary excretion of T_4.

The results in the present study suggest that abnormalities in thyroid histology and circulating thyroid hormone concentrations exist in juvenile alligators on Lake Okeechobee, specifically in animals collected from sites of historical environmental contamination. Thus, thyroid gland disruption, structural and functional, in alligators appears to have an environmental etiology. In order to obtain a more accurate indication of thyroid activity, future research should include sampling at multiple points throughout the year and utilize responses at the level of both the thyroid and peripheral to the thyroid.

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Elizabeth A. Hewitt graduated in May 2000 from Maryville College. She graduated Magna Cum Laude with a B.A. in Biology and a minor in Chemistry. This paper is the result of Hewitt's 1999 summer research experience at the Department of Zoology, University of Florida.

D. Andrew Crain is currently Assistant Professor of Biology at Maryville College. He graduated with a Ph.D. from the University of Florida in 1997. Dr. Crain has 25 publications exploring normal and contaminant-altered endocrine systems. In 2000, he and Dr Louis Guillette co-edited a book entitled Environmental Endocrine Disruptors: An Evolutionary Perspective.

Mark P. Gunderson is a graduate student of Dr Lou Guillette's in the Department of Zoology, University of Florida. Mark graduated Magna Cum Laude during the spring of 1995 from St. Olaf College in Northfield Minnesota and was inducted into the Phi Beta Kappa honor society that same spring.

Louis J. Guillette, Jr. is Professor of Zoology and Alumni Distinguished Professor at the University of Florida. Dr Guillette received his doctorate in Comparative Reproductive Biology from the University of Colorado at Boulder in 1981. He has served as a science advisor to many US and foreign agencies regarding the developmental impacts of environmental contamination.