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Lipophilic organic compounds in lake sediment and American coot (*Fulica americana*) tissues, both affected and unaffected by avian vacuolar myelinopathy

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Abstract

Avian vacuolar myelinopathy (AVM) is a disease of unknown etiology, which has been diagnosed in a variety of birds from surface water reservoirs in the southeastern United States. Pathology suggests a natural or anthropogenic compound may be the cause of this disease. With the goal of identifying the toxicant that causes AVM, we qualitatively analyzed sediments and American coot (*Fulica americana*) tissues from reservoirs that were affected and unaffected by AVM using high-resolution gas chromatographic low-resolution mass spectrometry. Polychlorinated biphenyls (PCBs), octachlorodibenzo-*p*-dioxin, and biogenic and anthropogenic polycyclic aromatic hydrocarbons (such as retene) were the most abundant compounds in the sediment. Penta- and hexachlorobenzene, oxychlordane, *p,p′*-DDE, dieldrin, and polychlorinated biphenyls were the most abundant compounds in the avian tissues. None of these compounds were more abundant in the AVM affected sediments and tissues than in the unaffected media. Therefore, it is unlikely that any of these compounds are the cause of this avian disease.

Keywords: Avian vacuolar myelinopathy; Sediment; Avian tissues; Organic compounds

1. Introduction

Avian vacuolar myelinopathy (AVM) is a disease of the nervous system first observed in wild bald eagles (*Haliaeetus leucocephalus*) from western Arkansas in the fall and winter of 1994 (Thomas et al., 1998). The disease was later observed in American coots (*Fulica americana*) from the same region (Thomas et al., 1998). Affected birds may appear uncoordinated and may have erratic flight, swim tipped to one side, and have difficulty walking (Thomas et al., 1998; Larsen et al., 2002). AVM appears in the brain as a splitting of the myelin lamellae, resulting in the formation of vacuoles within the myelin sheaths (Thomas et al., 1998). The histological characteristics of AVM are similar to lesions that have been naturally and experimentally induced by certain chemicals, for example hexachlorophene (Norton and Cammer, 1984) and an unidentified exotic plant toxin (Van der Lugt et al., 1996). This observation suggests a natural neurotoxin or anthropogenic neurotoxicant may be the cause of

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this disease. However, chemical analysis of the tissues of affected birds for compounds known to cause vacuolar myelinopathy in birds and mammals, as well as specific metals and organochlorine compounds, has not helped investigators determine the etiology of this disease (Thomas et al., 1998).

AVM has since been diagnosed in birds from surface water impoundments, or reservoirs, in five southeastern States. Additionally, AVM has been confirmed in other species of birds, including three species of ducks. The disease has caused extensive morbidity and mortality of birds and represents the largest unknown cause of eagle mortality in the United States.

The aim of this study was to qualitatively identify the unknown toxicant that causes AVM by non-specific chemical analyses of the organic compounds present in sediment taken from AVM affected and unaffected lakes and in coot tissues from birds diagnosed as AVM positive and negative. The samples were extracted using organic solvents and analyzed using gas chromatographic mass spectrometry to identify compounds.

Structurally diverse compounds [for example triethyltin (Fleming et al., 1991), isonicotinic acid hydrazide (Carlton and Kreutzberg, 1996), and bromethalin (Dorman et al., 1992)] are known to cause AVM-like lesions, but many of these compounds have been eliminated as potential causes of AVM (Thomas et al., 1998). Therefore, rather than measure the amount of a pre-defined set of chemicals, in this study, we have used mass spectrometry to identify a broad range of compounds. We focused on hydrophobic organic compounds, because myelin is rich in lipids and can accumulate hydrophobic compounds. Also, the bioaccumulation and bio-concentration of hydrophobic compounds can result in a relatively high concentration in animals, such as birds, that occupy the higher trophic levels in the food web. Because this was an initial study of the hydrophobic compounds present, the identified compounds were not quantified. Instead, the normalized peak areas were used to qualitatively determine their abundance. We assumed that the unknown toxicant would be among the most abundant compounds found in the affected sediment and bird tissues and that this toxicant would be found at relatively high levels in the affected sediment and tissues but at relatively low levels in the unaffected sediment and tissues.

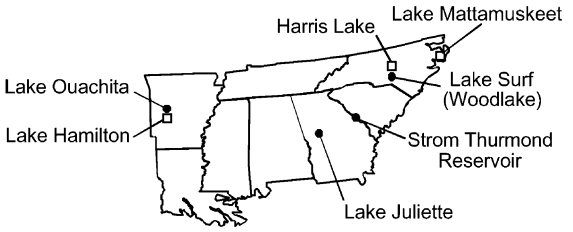

Fig. 1. AVM positive (●) and AVM negative (□) sampling locations in the southeastern US; disease status refers to condition of birds collected at these lakes in winter 2000, except for Lake Mattamuskeet which was not sampled until winter 2001.

2. Methods and materials

2.1. Study sites

The lakes that were sampled for sediments and coots are shown in Fig. 1. Four lakes were selected because of the documented presence of AVM in birds during the period of sample collection. Surveys of chemicals historically or currently used in the watersheds of AVM-positive reservoirs were conducted by contacting those responsible for their management. The few compounds identified as being used at all the sites were those commonly used throughout the southeastern US for submerged aquatic or littoral vegetation management (making them unlikely candidates as a causative agent for a disease apparently present at only a few locations). The four lakes were: Lake Ouachita (OUA, 34° 36′ N, 93° 20′ W), near Hot Springs, Arkansas, a 40 000-acre reservoir used for flood control, hydropower production, recreation, water supply, and environmental resources management; Lake Juliette (LJ, 33° 03′ N, 83° 48′ W), near Macon, Georgia, a 3500-acre reservoir used as a source of cooling water for a coal-fired power plant and for hunting and fishing; Strom Thurmond Reservoir (also known as Clarks Hill Lake) (ST; 33° 42′ N, 82° 14′ W), a 70 000-acre impoundment of the Savannah River along the Georgia/South Carolina border which is managed for flood con-

trol, hydropower production, recreation, water supply, and fish and wildlife; and Lake Surf (also known as Woodlake) (WL, 35° 14′ N, 79° 12′ W), a 1100-acre reservoir created in 1973 as part of a country club. The three reference (AVM negative) lakes were: Lake Hamilton (HAM, 34° 26′ N, 93° 05′ W), connected to Lake Ouachita), Arkansas, a 7200-acre reservoir with similar uses; Harris Lake (HL, 35° 36′ N, 79° 57′ W), North Carolina, a 4150-acre impoundment created in 1983 as a cooling water source for a nuclear power plant and used for recreation and research; and Lake Mattamuskeet (LM, 35° 30′ N, 76° 15′ W), North Carolina, a 41 000-acre natural lake within a National Wildlife Refuge managed for migratory bird habitat and wildlife-oriented recreation and education.

2.2. Sediment collection and analysis

All sediment samples were collected between February and April of 2000 following confirmation of AVM presence (OUA, WL, ST and LJ) or absence (HL and HAM) in wild birds from each location that season. Confirmation of disease was via light microscopy performed by the Southeastern Cooperative Wildlife Disease Study (SCWDS), Athens, GA or the US Geological Survey National Wildlife Health Center (NWHC), Madison, WI. Birds diagnosed as AVM positive had vacuolization of the white matter of the brain consistent with the findings of Thomas et al. (1998).

Sediment was collected from three sites in each lake using a small, stainless-steel Ponar dredge. This sampling device collects approximately the top 5 cm of sediment. Sediment samples were taken in shallow water (less than approx. 2 m in depth) near the shore. Sample locations included sites where water birds were known to congregate (probably because of the presence of submerged aquatic vegetation) and sites representing water sources to the reservoirs (i.e. major tributaries). At AVM positive lakes, sample locations included areas where sick or dead birds were observed.

At each of the three sites in a lake, several grab samples were collected and homogenized to make a single sample for that site. The samples were stored in solvent washed glass jars at <0 °C. The samples were shipped frozen to Bloomington, Indiana, and stored at -30 °C. For extraction, approximately 20 g of each sample was thawed, mixed with anhydrous Na_2SO_4 to obtain a free flowing material, and extracted in a Soxhlet device for 24 h. Two different extraction solvents were used: Two sediment samples from each lake were extracted with methanol, and the third was extracted with 50% acetone in hexane. All solvents were from EM Science, Gibbstown, NJ. The sediment dry weight was determined gravimetrically before and after heating an aliquot of each sample at 75 °C for 48 h.

The extracts were evaporated using a Rapidvap device (Labconco, Kansas City, MO), re-dissolved in approximately 100–150 ml of hexane, and transferred to a separatory funnel. Fifty milliliters of water and 50 ml of a 50% NaCl solution were added to the funnel. The funnel was shaken for 3 min, and the water layer was drained into a large bottle. The hexane layer was drained through a funnel holding approximately 150 g of anhydrous Na_2SO_4 into a bottle. The water was poured back into the separatory funnel, another 50 ml hexane was added, and the procedure was repeated as described above. The two hexane fractions were combined, evaporated, and fractionated on a 20×1.9 cm I.D., 3% water-deactivated silica column with copper granules at the bottom to remove elemental sulfur. Three fractions of 50 ml each were collected using hexane (non-polar), 50% hexane in dichloromethane (semi-polar), and dichloromethane (polar).

Each fraction was evaporated, solvent exchanged into hexane, and transferred to a gas chromatography (GC) auto injector vial for gas chromatographic mass spectrometry (GC/MS) analysis. Samples were analyzed on Hewlett-Packard 6890 series gas chromatograph coupled to a Hewlett-Packard 5973 mass spectrometer with helium as the carrier gas. The mass spectrometer was operated in full-scan mode, using both electron impact ionization and electron capture negative ionization; the latter used methane as the reagent gas. The GC column was coated with DB-5-MS (60-m length$\times$250-μm I.D., 0.25-μm film thickness, from J&W Scientific, Folsom, CA). The

temperature of the GC oven was programmed as follows: isothermal at 40 °C for 1 min, heated at 30 °C/min to 130 °C, heated at 3 °C/min to 241 °C, heated at 30 °C/min to 285 °C, and held at 285 °C for 35 min. The mass spectrometer was scanned between m/z 40 and 800.

2.3. Tissue collection and analysis

American coots were collected between November 1999 and February 2001 from Lake Surf (WL) and Lake Mattamuskeet (LM) (see Fig. 1). With the exception of coots for which analytical results are presented for brain composites, all birds analyzed from WL were diagnosed as AVM positive, and all birds from LM were diagnosed as AVM negative by the SCWDS or the NWHC as described above. For birds from which brains were analyzed, no diagnosis was made (the diagnosis required brain histopathology). Instead, they were presumed to be AVM positive or negative based on the documented disease status of cohorts caught from the same site at the same time.

The 27 birds analyzed in this study were collected either by shooting or by trapping. Four separate samples were prepared by compositing all soft tissues of four different birds (adipose, pectoral muscle, liver, kidney, lung and gastrointestinal tract and its contents; the brain tissues had previously been removed for AVM diagnosis). Two of these samples were from known AVM positive birds, and the other two were from known AVM negative birds. These mixed-tissue composites maximized sample mass. We also prepared two composite samples of livers (one sample from known AVM positive birds and the other from known AVM negative birds), two composite samples of adipose tissue (one sample from known AVM positive birds and the other from known AVM negative birds), and two composite samples of brains (one from presumed AVM positive birds and the other from presumed AVM negative birds, based on the disease status of birds collected at the same time and place). These individual tissue samples were composites of tissues from between two and five coots. After dissection, the coot tissues were stored in solvent washed glass jars at <0 °C and shipped frozen to Indiana University.

Depending on the amount of sample available, 3–25 g of tissue was extracted. The wet tissue was ground with approximately 130 g of anhydrous Na_2SO_4 and loaded into a Soxhlet extractor. The samples were then extracted with 50% acetone in hexane for 24 h. The extracts were reduced in volume using a Rapidvap device, solvent exchanged into 60% cyclohexane in dichloromethane, and centrifuged to remove any water or non-soluble components that may have precipitated. The lipid concentration of each extract was determined by gravimetric measurement. Most of the lipids were removed using a gel permeation chromatography system consisting of a 100×2.5-cm I.D. glass column packed with SX-3 Bio-Beads (Bio-Rad Laboratories, Hercules, CA). The eluent was 60% cyclohexane in dichloromethane, with a flow rate of 5 ml/min. The lipid-containing fraction was collected between 0 and 40 min, and the analyte-containing fraction was collected between 40 and 135 min. The samples were then reduced in volume using a Rapidvap device and fractionated on a 20×1.9-cm I.D., 3% water-deactivated silica column. Three fractions of 75 ml each were collected using hexane, 50% hexane in dichloromethane, and dichloromethane. Each fraction was reduced in volume by rotary-evaporation, transferred to a GC autoinjector vial, and analyzed by GC/MS using the parameters described above for the sediment analysis.

A procedural blank was extracted and analyzed in parallel with every batch of sediment and tissue samples. The procedural blank consisted of approximately 130 g of Na_2SO_4. In addition, laboratory and field blanks from the US Fish and Wildlife Service were analyzed. These blanks consisted of solvent washed glass jars that were exposed either to the laboratory or field atmosphere for 10–15 min. The jars were analyzed by rinsing the insides with 50% hexane in acetone, reducing the solvent volume using a stream of nitrogen, and analyzing the blank samples by using the GC/MS parameters described above.

2.4. Data analysis

We assumed that an unknown toxicant would be present in all three sediment samples from a

given lake or that the toxicant would be at relatively high concentrations at one or two locations. This assumption is reflected in our data analysis scheme. The total ion chromatograms for each of the three sediment samples from each lake were overlaid to select peaks that were present in all three samples, abundant but found in two samples, or very abundant but found in just one sample. This process selected approximately 20–30 GC peaks per lake. These lake-specific data were then compared to each other, and compounds that were present in a majority of the lakes were selected for identification. At this stage, we found the electron impact mass spectral library from NIST (1998) and the electron capture negative ionization library from Stemmler and Hites (1988) to be particularly helpful.

The coot tissue samples generally contained a smaller number of peaks than the sediment; therefore, for each coot sample, the 10–20 most abundant peaks in the total ion chromatogram were selected. The data from the tissue samples were then compared, and compounds that were present in a majority of the samples were selected for identification.

Some compounds were excluded from the data analysis scheme. Compounds that were present in the blanks were not included. Saturated and unsaturated hydrocarbons, elemental sulfur, and fatty acid esters were excluded. These compounds were found in both AVM positive and AVM negative samples. Some compounds in the sediment were only extracted by either methanol or hexane/acetone. All of these compounds could be excluded, either because they belonged to one of the compound classes described above or because they fit the criteria described below for compounds detected by electron capture negative ionization.

Electron impact mass spectrometry ionizes all compounds about equally, giving a good estimate of the relative amount of each compound in the sample. Electron capture negative ionization mass spectrometry, on the other hand, is 10–100 times more sensitive towards compounds containing functional groups (such as halogens) that can readily capture electrons. Therefore, if an unidentifiable compound was found under electron capture negative ionization conditions but not under electron impact conditions, we considered it to have a relatively low abundance, and it was excluded from further identification. None of the excluded compounds were present in the AVM positive samples but absent in the AVM negative samples; thus, none of the excluded compounds could be the cause of AVM.

The sediment data were normalized to the dry weight, and the tissue data were normalized to the mass of extracted lipids. The normalized abundances of each compound in each of the three sediment samples from each lake were averaged. Because quantitation standards were not used, concentrations are not reported. Instead, the approximate relative abundance of each compound is represented relative to the most abundant compound in the data set by the following scheme: $++++$, abundance $>50\%$; $+++$, abundance between 20 and 50%; $++$, abundance between 5 and 20%; $+$, abundance below 5%; and $-$, not observed.

3. Results and discussion

Several biogenic and anthropogenic polycyclic aromatic hydrocarbons (PAH), were found in the sediment samples taken from both the AVM positive and AVM negative lakes (see Table 1). Sedimentary PAHs have anthropogenic (such as the incomplete combustion of fossil fuels) and natural sources (Hites et al., 1980). The latter include the seepage of petroleum, the products of forest fires, and the transformation, within the sediment, of natural precursors found in terrestrial plants. In this study, PAHs from two different natural precursors were observed. The first group is derived from abietic acid, a constituent of plant resins, particularly conifers (Wakeham et al., 1980); see Fig. 2 for structures. The decarboxylation and aromatization of abietic acid gives, sequentially, dehydroabietin, 1,2,3,4-tetrahydroretene, and retene. These compounds were identified from their known mass spectra (Wakeham et al., 1980; Wen et al., 2000). As an example, the mass spectra of the tetrahydroretene found in the sediment and of the authentic compound are shown in Fig. 3. Incidentally, we had originally identified this compound as a diisopropylbiphenyl isomer based on a

Table 1
Date of collection, average wet weight, average percent dry weight, and the dry weight normalized relative abundance for each compound found in the sediments of the southeastern US reservoirs. See Fig. 2 for structures

Lake[a]	AVM positive				AVM negative	
	OUA	LJ	ST	WL	HAM	HL
Date	4/6/00	4/12/00	2/2/00	2/9/00	4/6/00	4/4/00
Wet wt. (g)	21.4	22.1	21.9	20.2	22.1	20.8
% dry wt.%	35	55	59	53	52	42
Dehydroabietin	+++	+	+	++++	+	+
Tetrahydroretene	+++	+	+	+++	+	+
Retene	++	+	+	+++	+	++
TMTHC-A[b]	++	++	+	++	−	++++
TMTHC-B[b]	+++	+++	++	−	++	++++
Perylene	+++	++	++	+	++	++
PAH $C_{16}H_{10}$[c]	+	+	−	+	+	+
PAH $C_{18}H_{10}$[d]	+	+	−	+	+	+
PAH $C_{20}H_{12}$[e]	+	+	+	+	+	+
Penta-PCB[f]	+	+	+	+	+	+
Hexa-PCB[f]	+	+	+	+	+	+
OCDD[g]	+	+	−	+	+	+

[a]OUA, Lake Ouachita; LJ, Lake Juliette; ST, Strom Thurmond Reservoir; WL, Lake Surf (Woodlake); HAM, Lake Hamilton; HL, Harris Lake; [b]trimethyltetrahydrochrysenes; [c]likely to be fluoranthene and pyrene; [d]likely to be benzo[*ghi*]perylene; [e]likely to be benzo[*a*]pyrene or an isomer; [f]polychlorinated biphenyls; [g]octachlorodibenzo-*p*-dioxin.

reference mass spectrum of that compound found in the NIST library (NIST, 1998; Peterman and Delfino, 1990); see Fig. 3, bottom. The similarity of this mass spectrum to that of the tetrahydroretene is remarkable, given the different carbon skeletons of these two compounds. However, based on differences in GC retention times, we were able to confirm that our unknown was not a diisopropylbiphenyl.

The trimethyltetrahydrochrysenes (labeled TMTHC-A and -B in Table 1) were also identified from their known mass spectra (Wakeham et al., 1980); see Fig. 2 for structures. These compounds are derived naturally from amyrins (Laflamme and Hites, 1979), major components of plant waxes. Perylene may have either natural or anthropogenic sources or both (Bouloubassi and Saliot, 1993), but in this case, we suspect that most of this compound occurs naturally. The PAHs with empirical formulas $C_{16}H_{10}$, $C_{18}H_{10}$, and $C_{20}H_{12}$, probably have anthropogenic sources due to the combustion of fossil fuels; for example, $C_{20}H_{12}$ is the elemental composition of benzo[*a*]pyrene, a carcinogenic compound produced by burning almost anything.

The penta- and hexachlorinated polychlorinated biphenyl (PCB) homologues certainly have anthropogenic sources. In fact, penta- and hexachlorinated substituted PCBs are usually the most abundant PCB homologue groups found in sediment (Mudroch et al., 1989). Octachlorodibenzo-*p*-dioxin (OCDD) is usually thought to be from anthropogenic sources, such as the combustion of municipal solid waste (Hites, 1990), but there is a growing consensus that it could also have natural sources (Green et al., 2001). In either case, OCDD is usually the most abundant dioxin homologue found in sediment (Baker and Hites, 2000). All of the compounds in Table 1 were found in the sediment from AVM positive and negative lakes. Thus, it is unlikely that these compounds are related to the etiology of the disease.

The PAH detected in the sediments were not present in the bird tissue samples (see Table 2) presumably because they were readily metabolized (Escartín and Porte, 1999). On the other hand, penta- and hexachlorobenzene (HCB) were present in most of the samples. In addition, the pesticide metabolites, oxychlordane (from chlordane), *p,p′*-DDE (from DDT), and dieldrin (a metabolite of

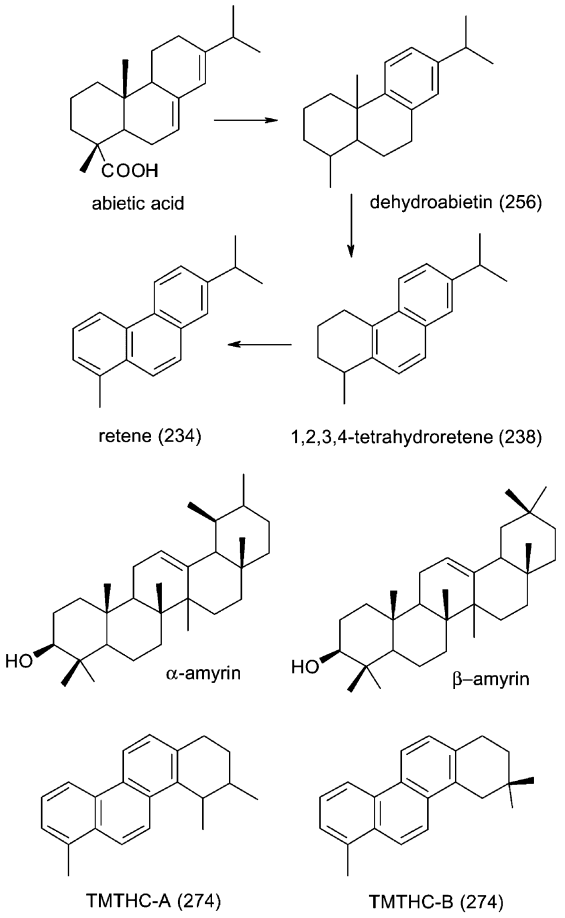

Fig. 2. Structures (and molecular weights) of compounds found in the various lake sediment samples; see Table 1.

aldrin as well as an insecticide on its own) were found in the AVM positive and AVM negative birds (see Table 2). Penta- through deca-chlorinated PCBs were also observed in both sets of bird tissues. PCBs were the most abundant group of compounds found, which is a common observation (Dodder et al., 2002). The brain tissues of presumed AVM positive and negative birds contained relatively less organochlorine contaminants than the other tissue types, perhaps due to the more polar nature of the lipids found in the brain versus adipose tissue (Dewailly et al., 1999). Although the organochlorine compounds found in these samples have been implicated in a number of wildlife diseases (Hoffman et al., 1990), they are probably not a cause of AVM because our data show that these compounds are found at approximately equal abundances in the AVM positive and the AVM negative birds.

Neither the sediment data nor the tissue data show any compounds that are present in all of the AVM positive lakes but not present (or of low relative abundance) in the AVM negative lakes. This study was, therefore, unsuccessful in identifying a compound responsible for AVM. There are several reasons for this failure: The extraction procedures and GC/MS parameters were optimized to measure non-polar organic molecules with molecular weights between approximately m/z 40 and 600. This represents a very broad

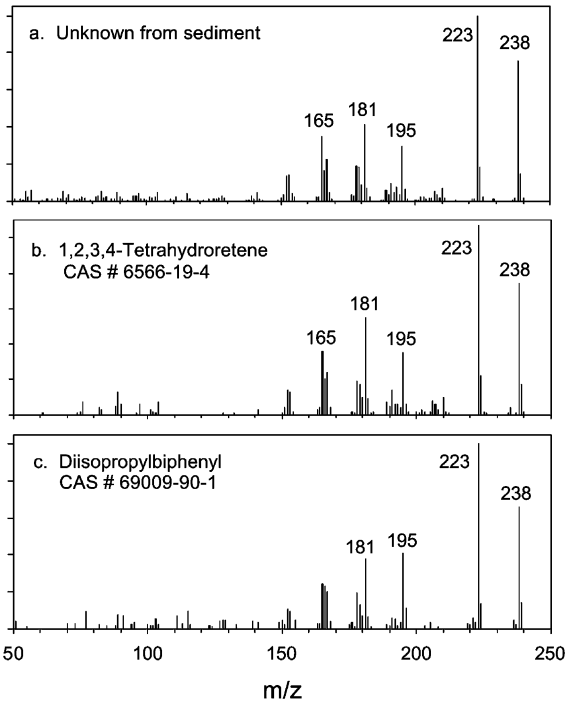

Fig. 3. Identification of 1,2,3,4-tetrahydroretene in sediment samples. Mass spectra of (a) a typical unknown peak from a sediment; (b) 1,2,3,4-tetrahydroretene (Wakeham et al., 1980; Wen et al., 2000); and (c) diisopropylbiphenyl, obtained by analysis of the commercial mixture Sure Sol 330 (Peterman and Delfino, 1990). The GC retention time of the unknown was 5 min later than that of the latest eluting diisopropylbiphenyl isomer.

Table 2
Tissue type, sampling location, date of sampling, number of coots in composite, sample wet weight, percent lipid, and the lipid weight normalized relative abundance for each compound found in the tissues of AVM positive and AVM negative birds.

	AVM positive					AVM negative				
Tissue	Comp.[a]	Comp.	Brain	Liver	Fat	Comp.	Comp.	Brain	Liver	Fat
Lake[b]	WL	WL	WL	WL	WL	MT	MT	MT	MT	MT
Date	11/99	11/00	11/00	11/00	11/00	2/01	2/01	2/01	2/01	2/01
Num.	1	1	4	5	5	1	1	2	5	2
Wet wt.% (g)	24.2	24.3	8.4	20.9	6.7	25.5	24.8	3.7	20.9	3.2
% lipid	13	7	7	5	82	32	31	10	5	100
PnCB[c]	+	+	−	+	+	+	+	−	+	−
HCB[d]	+	+	++	++	++	+	+	+	++	++
OxCHL[e]	+	++	−	++++	+	−	−	−	++++	+
p,p′-DDE	++	++	−	++	+	+	+	−	++	+++
Dieldrin	−	++	−	++	−	−	−	−	+++	+
Penta-PCB	−	++	++	−	+	−	+	++	+	++
Hexa-PCB	+	+++	++	+++	++	+	+	+++	++	++++
Hepta-PCB	++	+++	+	++	++	+	+	++	++	++++
Octa-PCB	+	+	−	+	++	+	+	+	++	++++
Nona-PCB	+	+	−	−	+	−	+	−	+	+++
Deca-PCB	+	+	−	−	−	−	+	−	+	++

[a] Composite of several tissues: see text.
[b] WL, Lake Surf (Woodlake); MT, Lake Mattamuskeet.
[c] PnCB, pentachlorobenzene.
[d] HCB, hexachlorobenzene.
[e] OxCHL, oxychlordane.

range of natural and anthropogenic compounds. However, these methods will not detect compounds such as proteins, metals, or polar organic compounds. A compound from one of these classes could be the neurotoxin or neurotoxicant responsible for AVM [some specific compounds from these classes were analyzed in AVM positive birds by Thomas et al. (1998); none were found at significant levels]. Alternatively, the toxicant could be a non-polar organic compound present at such low concentrations that it cannot be detected in the presence of a background of abundant naturally occurring compounds.

Further experiments are underway to learn how the birds are being infected, and these experiments may aid in the identification of the neurotoxin or neurotoxicant. This work includes sentinel bird studies (i.e. release of unaffected birds at AVM positive and AVM-negative lakes to track disease on-set and duration), feeding studies (on-going attempts to reproduce the disease by feeding water, sediment, algae, vascular plants, forage fish, and invertebrates from AVM-positive lakes to several species of birds), aquatic plant inventories, and zooepidemiological studies.

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