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Epidemiologic studies of agents present in the environment seek to identify the extent to which they contribute to the causation of a specific toxic, clinical, or pathological endpoint. The multifactorial nature of disease etiology, long latency periods and the complexity of exposure, all contribute to the difficulty of establishing associations and causal relationships between a specific exposure and an adverse outcome. These barriers to studies of exposure and subsequent risk assessment cannot generally be changed. However, the appropriate use of biological markers in animal species living in a contaminated habitat can provide a measure of potential damage from that exposure and, in some instances, act as a surrogate for human environmental exposures. Quantitative predictivity of the effect of exposure to environmental pollutants is being approached by employing an appropriate array of biological end points.

Human exposure to environmental pollutants is often exceedingly complex. Sources of exposure are multiple, and the importance of a particular source may depend on individual practices. Humans are highly mobile, and direct study, particularly when physiological testing is required, is generally expensive and logistically difficult. For these reasons, a simpler animal model which provides a parallel biological marker in humans would be useful.

Small mammals have frequently been used to monitor the presence of metals and other contaminants in the environment. Such biomonitoring can be essential for determining bioavailability and resultant biological effects under natural conditions. Small mammals can be particularly effective biomonitors of soil contaminants if the species used are abundant, easily caught, do not migrate long distances, and have well documented food habits

(1). Good biomonitors are in equilibrium with the environment and should show a graded response to a range of pollutants.

Data are sparse on the amounts of hazardous substances released to the environment. Measuring concentrations of these substances in soil, plants, and animals would require an exhaustive monitoring program which would be costly, especially since many chemicals will not be present as the parent compound in living organisms due to their rapid metabolism. The important question is not whether these substances are present, but rather whether these substances enter the biota in chemical forms and concentrations that will result in an adverse biological effect. Thus, monitoring the biological availability of these substances in biota becomes a critical parameter for determining whether they have potential for being hazardous and whether they could result in significant exposures to humans (2).

The estimation of the risk resulting from exposure to environmental pollutants requires knowledge of the dose and pharmacokinetics of the chemicals in the animal. A systemic dose in an individual can be obtained by quantitation of the chemical or its metabolites in body fluids such as blood, urine, feces, sputum, or milk, however, a major drawback to this approach is that the analysis must be performed shortly after exposure, that is, prior to the clearance of the metabolites from the body. Alternative procedures for monitoring exposure to hazardous substances are needed. In particular, new methods are needed for measuring reaction products of chemicals, such as metabolites and adducts, in animals and humans that correlate with patterns of associated biological change and pathological conditions (3,4).

Our approach to detecting exposure to hazardous chemicals in the environment, and to estimating the potential for subsequent adverse effects, is to monitor biological endpoints in wild animals. Biological endpoints include indicators that: (a) quantify exposure by measuring chemical interaction with critical molecular targets (adducts in nucleic acids and proteins); (b) measure alterations in specific, sensitive, and critical physiological and biochemical processes (detoxifying enzyme activities and metal-binding proteins); and, (c) monitor early expression of cellular dysfunction (neoplasia or loss of immune competence). Using this approach the organism is seen to function as an integrator of exposure, accounting for abiotic and physiological factors that modulate the dose of toxicant taken up from the environment. The biological marker is used to detect the response of the organism to the environmental insult. Because of the often long latent period between exposure and subsequent irreversible deleterious impact on health, it would be desirable to have sensitive biological indicators that could detect exposure early enough so that preventive or remedial measures could be taken. Three elements are critical to our approach: (a) marker selection based on toxicological mechanisms; (b) field studies to establish correlations between environmental contamination and markers; and, (c) laboratory confirmation of causal relationships between exposure, biological markers, and adverse effects.

Biological monitoring can be used as a cost-effective survey of contaminated sites, for routine monitoring of uncontaminated sites,

to provide evidence for contaminant impact on biota, and to establish priorities for site cleanup.

A suite of biomarkers that we currently use to monitor for exposure to selected environmental pollutants, is shown in Table I. Information obtained upon monitoring the response of these biomarkers is compared to long-term adverse effects in the organism (reduced fecundity, decreased disease resistance, and tumor formation).

Table I. Biological Markers of Exposure and Bioavailability of Environmental Contaminants

Environmental Pollutant	Biological Marker	Reliability Index*
A. Toxic Metals:		
Cu, Hg, Ag,	DNA integrity/m^5dCyd	s
Zn, Cd, Pb,	metal-binding proteins	s,d
	porphyrin biosynthesis	s,d,p
	immune response	s
	xenobiotic metabolism	s
B. Organic Cmpds:		
1. PAH's	DNA/hemoglobin adducts	s,d,p
	xenobiotic metabolism	s,d
	immune response	s
	DNA integrity/m^5dCyd	s
2. PCB's & TCDD	xenobiotic metabolism	s
	porphyrin profile	s
	immune response	s
	DNA integrity/m^5dCyd	s
	DNA/hemoglobin adducts	s,d

* s: signal of potential problem
d: definitive indicator of type or class of pollutant
p: predictive indicator of long-term adverse effect

Studies with Cellular Biomarkers

DNA Modification. As a result of exposure to hazardous substances in the environment, it is not unreasonable to expect that damage to DNA may occur that goes uncorrected, with subsequent adverse effects to the organism.

The rationale underlying the strategy of chemical dosimetry by determining levels of compounds which become covalently bound to cellular macromolecules is based on our understanding of the mode of action of genotoxic compounds (5-7). The metabolism of chemicals is a relatively complex phenomenon and detoxification by cellular mechanisms is not always complete, sometimes resulting in highly reactive electrophilic metabolites. These metabolites can

undergo attack by nucleophilic centers in macromolecules such as lipids, proteins, DNA and RNA which often results in cellular toxicity. Binding with DNA, however, can cause formation of altered bases that can be repaired, be innocuous, or result in alterations which become fixed and are transmitted to daughter cells. Current research suggests that reaction of chemicals with DNA and the ensuing changes which result can cause cancer.

Given that genotoxic agents exert their activity through irreversible reactions with nucleophilic atoms (adducts), the amount of such reaction products will be proportional not only to the in vivo concentration of the electrophile, but also to the time this concentration is maintained. Therefore, the amount of metabolite bound to cellular DNA (in vivo dose), provides a reliable dosimetric basis upon which to assess the risk connected with exposure to a genotoxic compound (3,7,8).

It should be noted that the detection and quantitation of DNA adducts is not a simple task because there are few analytical techniques currently available. The reasons for this are: (a) the assay often has to accommodate the unique chemical and/or physical properties of the individual compound or its adduct; (b) the percentage of total chemical that becomes attached to the DNA in the target tissue is quite small because most of it is usually detoxified and excreted; (c) not all adducts that form between the genotoxic agent and DNA are stable or are involved in the development of subsequent deleterious events in the organism; and, (d) the amount of DNA available for analysis from the target tissue or organ is often quite limited.

Recently our laboratory demonstrated an alternative method to radiometric, immuno, or postderivatization detection of the polycyclic aromatic hydrocarbon, benzo[*a*]pyrene (BaP) binding to DNA (9). Essentially, the technique consists of the acid-induced removal of the diolepoxide adduct of BaP from the macromolecule as the strongly fluorescent free tetrols which are then separated and quantitated by fluorescence/HPLC analysis. The resulting chromatographic profile can be used to establish the stereochemical origin of the diolepoxide involved in adduct formation (10).

Benzo[a]pyrene-DNA Adduct Detection. Our initial research with the fluorescence/HPLC technique has demonstrated that we can detect, identify, and quantitate, subsequent to several routes of exposure, the binding of BaP with cellular DNA of mice (10-12) and fish (13). The detection and quantitation of adduct formation in fish exposed to BaP demonstrate that aquatic species are similar to other organisms in that they possess cellular enzymes capable of metabolizing BaP to the ultimate carcinogenic form of the chemical, the diolepoxide. A comparison of BaP-adducts formed with the DNAs of mice and fish demonstrate similarities (Table II), and suggest the feasibility of extrapolation of exposure data, based on the detection and quantitation of DNA adduct levels, between species.

Field Studies with Beluga Whale. Large quantities of PAHs, including the strongly carcinogenic BaP, have been reported in the main habitat of the St. Lawrence beluga whale population. These compounds are the probable cause of the increased frequency of

Table II. Comparison of DNA Adduct Formation in the Mouse and Bluegill Sunfish after an Acute Exposure to Benzo[a]pyrene

Species	Macromolecule	DNA Adduct Formation* I-1	II-2	%I-1
Mice	Skin DNA**	588	220	73
Fish	Liver DNA***	928	127	88

* DNA adduct formation determined 72 hours subsequent to BaP exposure and expressed as nanograms of tetrol I-1 or II-2 per gram of DNA. Data taken from (10-13).
** Topical application of BaP at 3 micrograms per gram body weight.
*** Intraperitoneal injection of BaP at 5 micrograms per gram body weight. Fish maintained at 20°C.

bladder tumors seen among aluminum workers from the same area (14), and since the compounds contaminate the beluga whale food chain, they are considered to be the etiological factor for the high frequency of tumors seen in this population (15).

Detectable levels of BaP-DNA adducts were found in the brains of beluga whales from the St. Lawrence estuary, while the occurrence of BaP-DNA adducts of the brain and liver of belugas taken from the Mackenzie estuary was not observed (Table III).

Table III. Detection of Benzo[a]pyrene Adducts in DNA of Beluga Whales (Delphinapterus leucas)

Sample	Tissue	BaP Adduct Formation binding*	level**
St. Lawrence Estuary			
#1	Brain	206	2.15
#2	Brain	94	0.98
#3	Brain	69	0.73
Mackenzie Estuary			
#1 - #4	Brain	none detected	
#1 - #4	Liver	none detected	

* BaP adducts to DNA expressed as nanograms of tetrol I-1 per gram of DNA. Data taken from (15).
** Level of adduct formation expressed as BaP adducts per 10^7 DNA nucleotides.

The data obtained are important for several reasons. First, they provide evidence that the whales from the St. Lawrence estuary had been exposed to BaP, and had metabolized it to the diolepoxide, which subsequently became covalently bound to the DNA of the brain tissue. Over the past several years a strong and convincing argument has developed for a causal relationship between the carcinogenic potency of BaP and the amount bound to an organism's DNA as a result of cellular metabolism (6,16). This is reinforced by the premise that the integrity of DNA is essential for survival. Alterations, if left uncorrected, could trigger a sequence of events that culminates in the appearance of an overt malignancy. Second, the level of adducts detected approaches that found in animals, both terrestrial and aquatic, exposed under controlled laboratory conditions to a carcinogenic dose of BaP (12,13,17,18). Third, cells in brain tissue are known to have slow turnover rates and lack or have very low capacity for excision repair of DNA damage. Therefore, significant accumulation of DNA adducts could occur in this tissue from long-term chronic exposure to environmental levels of BaP too low to induce neoplasia (19). It should be noted that the health of an animal may influence the formation of adducts. The whales from the Mackenzie estuary were hunted animals and not known to be diseased at the time of their capture, while those from the St. Lawrence were beached animals.

Non-adductive DNA Damage. Certain genotoxic compounds or agents such as metals, radionuclides, some chemicals, etc., do not covalently bind to DNA, but nevertheless induce damage. If this damage is expressed as strand breakage (or the potential for strand breakage), it can be detected by measuring the rate of alkaline-induced strand separation (20).

Analysis by a modified alkaline unwinding assay (21) demonstrated that chemically induced DNA strand breakage could be detected in bluegill sunfish and fathead minnows exposed to chronic, low levels of BaP. This technique has been used to determine the relative DNA damage, as measured by strand breakage, in bluegill sunfish taken from several sites in White Oak Creek, which is a small stream that flows through the Department of Energy reservation at the Oak Ridge National Laboratory and terminates in White Oak Lake. Biomonitoring activities are being conducted in this system (27).

The data in Table IV. show that the integrity of the DNA of the fish in the White Oak Creek System, except for those at the 3.0-3.4 km site, is similar to that of fish taken from a control site (Brushy Fork Creek, which is located some distance from the ORNL reservation) for both the Spring and Fall collection times. Additonal studies with fish collected at the 3.0-3.4 km site are in progress. These data, although preliminary, indicate the potential for measuring DNA damage as a general biological marker of exposure of indigenous animals to contaminants that occur in their environment.

Hemoglobin Modification. Because it meets a number of essential requirements, hemoglobin has been proposed as an alternative cellular macromolecule to DNA for estimating the in vivo dose of

Table IV. Relative DNA Strand Breaks in Bluegill from the White Oak Creek Watershed

Site	n*	
	Spring 1987	Fall 1987
W.O.C.(3.0-3.4 km)	4.77	2.01
W.O.C.(2.6 km)	0.68	0.03
White Oak Lake	0.75	1.13
Brushy Fork Creek	0.91	1.15

* $n = (\ln F_s / \ln F_c) - 1$ = number of breaks per alkaline unwinding unit of DNA (see refs 20,21). F_s represents fraction of DNA in double stranded form from fish at sample sites listed. F_c represents fraction of DNA in double stranded form from fish at Melton Branch, a small stream that enters the White Oak Creek (W.O.C.) at 2.5 km. A minimum of five fish were analyzed at each site.

chemicals subsequent to exposure (22-25). First, it has reactive nucleophilic sites and the reaction products with electrophilic agents are stable. Over fifty compounds to date have been shown to yield covalent reaction products with hemoglobin in animal experiments. These compounds include representatives from most of the important classes of genotoxic chemicals currently known. No tested mutagenic or cancer-initiating compound has failed to produce covalent reaction products with hemoglobin. Secondly, hemoglobin has a well established life-span, is readily available in humans and animals, and its concentration in an individual is not subject to large variation. Thirdly, modification of hemoglobin has been shown to give an indirect measure of the dose of reaction product that binds to the DNA in the target cells.

Biomonitoring of hemoglobin-bound metabolites represents a novel approach to the estimation of exposure to potentially harmful chemicals (26). Although these adducts have no putative mechanistic role in carcinogenesis, they do relate quantitatively to exposure and to activation since they may approximate the systemic dose (in vivo dose) of a chemical. They can be a measure of the chemical's genotoxic potency as well. Furthermore, since the adducts which form with hemoglobin are stable and have life-spans equal to that of the circulating erythrocyte (23), quantitation of these adducts can be used to integrate the dose obtained from chronic low-level types of exposure which most likely occur in chemically-polluted environments.

Field Studies with Terrestrial Animals. A floodplain on East Fork Poplar Creek (EFPC) was selected as a study site. EFPC originates at New Hope Pond, a point source of industrial pollution, and the study site is about 4 kilometers downstream. The floodplain is low and the creek periodically overflows, depositing sediment. There

is abundant vegetation. Measured BaP concentrations in the soil was 70 nanograms/gram, although 10 times this amount have been reported. Concentrations in plants was less than 1/10th of that in the soil. Small animals were trapped at this site to determine the practicality of using hemoglobin as a suitable biological marker of BaP pollution.

Preliminary data (Table V) indicate detectable concentrations of BaP adducts with hemoglobin were present in some individuals of species which have intimate contact with the sediment or soil. The shorttail shrew burrows into the ground and eats earthworms and insects, while muskrats feed on invertebrates found in the creek bed and browse on muddy vegetation along the creek bank. BaP adducts were not detectable in herbivores such as the white-footed mouse.

Table V. Concentration of Benzo[a]pyrene Adducts in the Hemoglobin of Animals on the EFPC Floodplain

Species	Number Analyzed	BaP-hemoglobin Adducts*
White-footed mouse (*Peromyscus leucopus*)	15	0.00
House mouse (*Mus musculus*)	1	0.00
Shorttail shrew (*Blarina brevicauda*)	2	0.35
Black rat snake (*Elaphe obsoleta obsoleta*)	1	0.00
Snapping turtle (*Chelydra serpentina*)	1	0.16
Norway rat (*Rattus norvegicus*)	1	0.15
Muskrat (*Ondatra zibethica*)	6	0.23

* BaP adducts to hemoglobin expressed as nanograms of tetrol I-1 per gram of hemoglobin. Data taken from (27).

BaP-hemoglobin Adducts in Humans. Human daily exposure to BaP increases dramatically in smokers, as one cigarette contains between 10 and 50 nanograms of BaP. Since the life-span of a human red blood cell is several months, continual exposure to BaP at one or more packs of cigarettes per day should result in a measurable steady state concentration of BaP-hemoglobin adducts. A preliminary study was conducted in a limited population of humans to determine whether BaP-hemoglobin adducts could be detected. The data in Table VI (methodology according to (25,26)) show that adducts were present in five out of six individuals who smoked while similar analyses of non-smokers gave lower levels relative to smokers. These data demonstrate the feasibility of the technique,

however, intrinsic factors such as age, sex, health, and nutritional status, as well as extrinsic factors such as the presence of other chemicals, may influence the pharmacokinetics of BaP metabolism, and the incidence of adducts with hemoglobin.

Table VI. Benzo[a]pyrene Adducts in Hemoglobin of Smokers and Non-smokers

Sample No.	Sex	Age	Race	Smoking Habit*	BaP-hemoglobin Adducts**
1	m	23	C	none	64
2	m	44	B	none	24
3	f	54	C	20	nd
4	f	56	C	none	69
5	m	36	O	none for 8 yrs	73
6	m	40	C	none for 15 yrs	nd
7	f	28	C	none	nd
8	m	42	C	20	24
9	m	49	O	40	159
10	m	27	B	none	129
11	f	56	C	none for 1 yr	1402***
12	m	49	C	20	468
13	f	50	C	40	272
14	m	57	C	40+	229

* Cigarettes per day.
** BaP adducts to hemoglobin expressed as picograms of tetrol I-1 per gram of hemoglobin; nd: none detected.
*** Numerous fluorescent peaks present in chromatogram in addition to tetrol I-1 peak.

Xenobiotic Metabolism. Xenobiotics in the environment, if taken up by an organisms, may induce microsomal enzymes (mixed function oxidases, MFO's) that can detoxify many of these compounds (28). The MFO's catalyze a variety of reactions, including aromatic and aliphatic N-hydroxylation, N-dealkylation, and O-dealkylation. Paradoxically, the MFO's may activate certain chemicals to their carcinogenic metabolite(s) (29). The biochemical systems in fish responsible for these types of reactions are similar to those found in mammalian species (30,31). The use of enzyme assays in biological monitoring programs was reviewed by Payne (32) for different phyla of aquatic animals and was found to be useful in detecting known point sources of pollution.

Field Studies in Aquatic Organisms. Activities of several MFO parameters have been measured in fish collected from stations along East Fork Poplar Creek (33). A multivariate discriminant analysis was used to examine the pattern of several measures of the MFO system (ethoxyresorufin deethylase, NAD reductase, NADP reductase,

cytochrome P-450, and cytochrome b_5 levels) in fish from the different stations. The statistical analysis (Figure 1) confirm that female bluegill sunfish at station no.1 (EFPC at New Hope Pond), a source of industrial pollution, exhibited a significantly different pattern of MFO responses than fish collected 20 kilometers down stream (station no. 5) or from a pristine stream (control). In addition, histopathological analysis of various organs from fish along EFPC showed higher levels of lesions in the fish closest to station no.1.

These results (Figure 1) suggest that the MFO enzyme system can be useful as a biological marker in a monitoring program if the response of the system to environmental and physiological influences such as temperature, season, food, hormonal (sex), etc., are taken into consideration.

Summary and Conclusions

Effective environmental management requires knowledge of the transport and fate of contaminants in natural systems (34). Small mammals can serve as indicators of the presence and bioavailability of contaminants and selected monitoring species can be used to measure the accumulation of contaminants. Furthermore, knowledge of food habits and habitat may indicate pathways of contaminants to organisms

Monitoring biological endpoints in wild animals can be used as an alternative to extensive sampling and chemical analyses, particularly where a large area is involved and the origin or extent of contamination is unknown or poorly defined. Evidence of exposure of biota as determined by biological monitoring could help to establish the need for cleanup.

Our laboratory has been interested in devising methods for detecting the exposure of living organisms to hazardous chemicals and physical agents in the environment. Initial studies with chemical dosimetry analysis began with the development of a fluorescence/HPLC assay for the detection and quantitation of BaP-DNA adducts with a sensitivity sufficient for detecting environmental exposures. Subsequent research demonstrated the applicability of this technique to animal models, both aquatic and terrestrial, under various experimental conditions. Furthermore, hemoglobin modification by chemicals was shown to be equivalent to DNA binding as a dosimeter.

Studies in aquatic organisms, both in the laboratory and in the field, have demonstrated the feasibility of measuring MFO enzyme activities associated with the metabolism of xenobiotics, such as BaP, in conjunction with non-adductive DNA damage as potentially useful biomarkers for exposure and bioavailability of environmental pollutants.

Other biological markers are currently being investigated for their capacity to detect and quantitate exposure to contaminants. These include: (a) parameters of the immune response (interferon induction, antibody-forming cells, and lymphocyte response to mitogens); (b) induction of metal-binding proteins; and, (c) minor nucleoside content of DNA.

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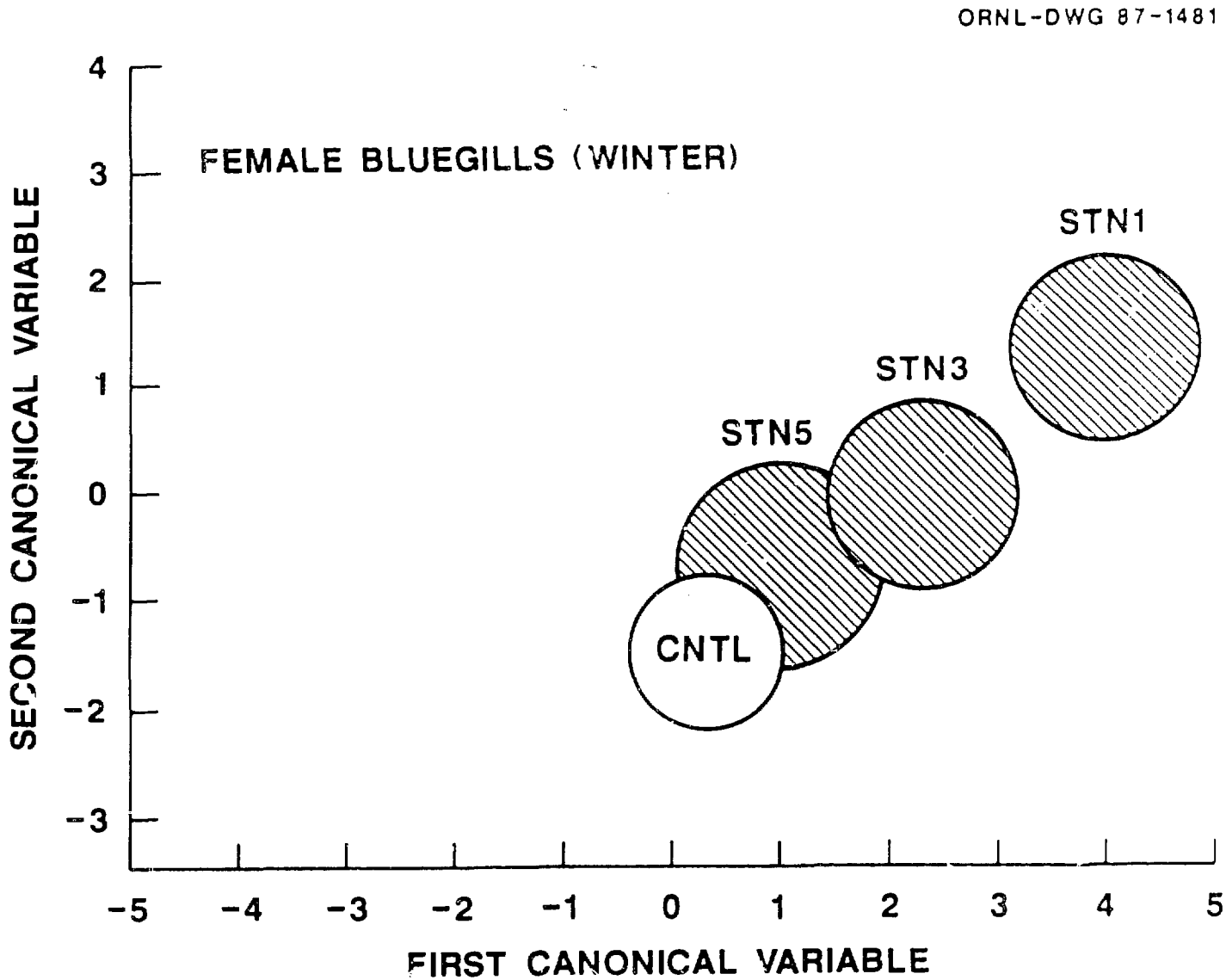
Figure 1. Segregation of integrated variable groups by discriminant analysis using several measures of the MFO system. Circles represent 95% confidence radii of control and respective test stations. Station no. 1 (New Hope Pond) is the headwaters of East Fork Poplar Creek; increasing station numbers are further down stream. Data taken from (33).


ORNL-DWG 87-1481
FEMALE BLUEGILLS (WINTER)
STN1
STN3
STN5
CNTL
SECOND CANONICAL VARIABLE
FIRST CANONICAL VARIABLE
4
3
2
1
0
-1
-2
-3
-5
-4
-3
-2
-1
0
1
2
3
4
5