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Temporal trend monitoring: Contaminant levels in tissues of Atlantic cod

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1 INTRODUCTION

The study of trends in trace contaminant concentrations in marine species has been of interest to the International Council for the Exploration of the Sea (ICES) since the early 1970s. The investigation of temporal trends in contaminant levels in fish, both as monitors of their environment and from a human health concern, is a topic under consideration by the ICES Working Group on the Statistical Aspects of Trend Monitoring (WGSATM) as a part of the Cooperative ICES Monitoring Studies Programme (CMP). Many problems were encountered during the first attempts by WGSATM to analyse the CMP data. In addition to the presence of outlying observations and significant differences between years in their coefficients of regression of (log) contaminant level(s) on (log) biological variable(s), many inconsistencies were found in the annual sampling structures (Anon., 1987). These often reflected the inability to replicate the size (length, age) structure characterizing earlier samples and the failure to obtain a sufficiently wide range in the covariables selected for study. These inconsistencies affected the annual regression lines, yielding, in many instances, insignificant regressions.

Further discussion within WGSATM identified many sampling and handling differences which probably arose from the too general nature of the past guidelines for trend monitoring. The Working Group emphasized the importance of essentially identical structures within all samples comprising a temporal trend study and emphasized that trend monitoring can only be done effectively if all steps of the study are thoroughly described and properly followed by all participants. Therefore, the WGSATM recommended that a series of leaflets be prepared giving specific details of sampling, handling, and analysis for each species being studied. The first leaflet (Uthe *et al.*, 1991) is a general introduction, describing the problems and requirements when monitoring temporal trends in contaminant levels in the marine environment, particularly biota.

This document describes the Canadian techniques used to study temporal trends in contaminant levels, both tissue concentrations and burdens (tissue concentration times the total weight of that tissue in the animal), in individual Atlantic cod (*Gadus morhua*) muscle and liver tissues over the period 1977-1985.

The decision to study Atlantic cod was based, *inter alia*, upon the importance of this species to Canada and the North Atlantic community, its widespread distribution within the ICES area, and the identification of a relatively discrete and stable cod stock in the southern Gulf of St. Lawrence. The procedural descriptions which follow are given only in outline form, along with the bases upon which such procedures were developed. Full details, including the statistical analyses carried out on the data, are given in the following references: Scott *et al.* (1978, 1981, 1983), Misra *et al.* (1988, 1989a,b,c), and Misra and Uthe (1985, 1986, 1987a,b). The chemical analytical procedures for the measurement of trace contaminants are not considered here, but are included in the above-mentioned references.

1.1 General Concept of Trend Analysis

Studies of temporal trends of contaminant levels, like all monitoring studies using fish, include a number of steps:

1) Development of a specific monitoring programme within a comprehensive quality management framework;
2) Selection and sampling of an appropriate population/stock for study;
3) Animal and tissue handling;
4) Measurement of biological and chemical parameters;
5) Data handling;
6) Statistical analysis; and
7) Interpretation.

Problems at any of the earlier steps can lead to difficulties at the later steps, resulting in less accurate, if not erroneous, information. Thus, it is essential that the programme be carried out under an appropriate quality management scheme (Uthe *et al.*, 1991) so that such problems can be avoided.

1.2 Quality Management of Trend Monitoring Programmes

It is obvious that any trend study must have clearly defined, realistic, specific, and, for the course of the study, essentially immutable goals upon which the quality management framework can be properly structured (Uthe *et al.*, 1991). Appropriate quality management will address each step of the study and have the authority to ensure that all procedures, including quality control procedures, are properly followed so that the types of errors may be identified and each kept within acceptable bounds, e.g., there must be insignificant drift in bias over time. A significant drift will be identified as a temporal trend in contaminant levels.

2 THE CANADIAN ATLANTIC COD STUDY: STEPS IN THE PROGRAMME

The procedures that were used in the Canadian Atlantic cod study were developed according to the following principles:

2.1 Define the Goal of the Study

The goal of the study was to determine the feasibility of monitoring temporal trends in common contaminant levels in a cod stock believed to be somewhat remote from direct coastal influences. Contaminants were selected from a suite as follows: (1) lead, mercury, cadmium, and polychlorinated biphenyls (PCBs) as primary contaminants undergoing changes in anthropogenic inputs, (2) zinc and copper as contaminants/essential nutrients which could possibly serve as indicators of unexpected metabolic alterations within the animal over time, and (3) arsenic, selenium, α-hexachlorocyclohexane (α-HCH), and hexachlorobenzene (HCB) as "opportunistic" contaminants, readily measured with a small work increment. While the initial protocol specified the measurement of all contaminants in both liver and whole fillets (red and white muscle combined), this was later modified due to financial constraints (Section 4 and Table 1 list the final contaminant/tissue combinations). Six biological variables were measured: length, age, liver weight, net weight (total animal weight minus liver weight), liver fat proportion, and muscle fat proportion.

2.2 Select a Species for Study

In addition to the considerations given above, cod was selected on the basis of its commercial importance, widespread prevalence, the available knowledge on the biology of the species, the relatively good relationship between size and age, the availability of large amounts of both muscle and liver tissue (so that pooling of tissues from several animals could be avoided), and the knowledge that hydrophobic contaminants concentrate in the liver of this species.

2.3 Select a Stable Stock

Of the four cod stocks thought to exist in the waters of the Nova Scotian area of Canada, that in the southern Gulf of St. Lawrence is believed to be the most isolated, least-mixing stock. The most favourable condition (genetically) in which a large, natural population exists is when it is subdivided into several partially isolated groups (stocks). Differences (for a quantitative measurement) between individuals within stocks would then be expected to be small compared to those between stocks. In addition, it is reasonable to expect that a stock will have a more homogeneous history of contaminant exposure compared to the population at large. Data on contaminant concentration levels and on biological variables from a sample of individuals of a stock are, therefore, likely to yield higher precision in statistical analyses for temporal trends than those from a sample of individuals from the entire population. This consideration is of particular importance in studies of temporal trends of contaminants, which, for practical reasons, must be based on a relatively small number (of the order of 25-60 (ICES, 1987)) individuals per sampling area and year.

2.4 Determine the Magnitude of Potential Change and Analytical Chemical Needs

It is important to ensure that the analytical method is capable of measuring concentrations down to the lowest expected levels so that very few "less than" values are reported. The first year's results of the Canadian programme (Scott *et al.*, 1978) showed that all contaminants selected for study should be suitable for trend studies, because in no instance was the level of the contaminant at, or near, the detection limit.

2.5 Select an Appropriate Sampling Time

A research population cruise on this fish stock was forecast for mid-September on an annual basis over the foreseeable future. Sampling simultaneously with fish stock assessment studies was judged to have a high potential benefit in terms of ultimately determining the effect of stock alterations on observed contaminant trends.

2.6 Develop an Appropriate Sampling Scheme based on Previous Knowledge of the Stock

Prior population cruises supplied information on which the size distribution of cod in single tows in the selected area could be calculated. This information was used to determine length strata, without gaps, to be filled with five fish each, selected on a first-come, first-chosen, basis from a single haul. The largest stratum was open ended. The smallest stratum contained fish just large enough to yield a sufficient quantity of tissue for analysis.

2.7 Select a Dedicated Sample Collector and Practice Sampling

One chemist was assigned to join all sampling cruises. Fish were selected on the vessel using length as the selection criterion and, as available, a few extra fish were added to each stratum to allow for rejection of abnormal animals. Each fish was tagged, bagged, and frozen as quickly

as possible on its side, as straight as possible for transport back to the laboratory. The chemist conducting this work was also experienced in working on a fishing research vessel and had practised the various shipboard and laboratory procedures previously.

2.8 Select the Analytical Laboratory

Ideally, the services of an analytical laboratory skilled in the handling of the selected species and the measurement of the selected contaminants in that species should be used. The Canadian laboratory has had much experience in measuring the selected contaminants in marine and freshwater biota, and is participating in a continuing programme concerning contaminants in fish and fish products. It was expected that the change in personnel over the period of the study would be minimal, if any. The laboratory also had extensive experience in fish preservation and changes occurring during cold storage. The importance of minimizing operator error by using one, highly trained and motivated, operator for each series of steps was recognized and was the approach used throughout the study, rather than attempting to train several operators to the required degree of operator comparability. Many problems in the handling of fish for trace contaminant studies and their resolution have been documented by Uthe and Chou (1988). It should be noted here that complete data were generated for each fish in the sample, i.e., there were no missing observations, because missing observations virtually destroy the possibility of using multivariate statistical analysis (Pimentel, 1979). Although procedures are available for estimating missing observations (e.g., Johnson and Wichern, 1988), it is moot whether anything is gained from their use.

2.9 Use a Statistician Experienced in Trend Analysis using Modern Statistical Methods

Statisticians (co-authors D.P.S. and R.K.M.) with long experience in fishery and contaminants research were involved in this study. The development/modification of multivariate statistical analytical programs was also required as a supplement to the univariate statistical analyses recommended in the ICES (1987) guidelines for analysing temporal trend monitoring data. Where contaminant variables are highly correlated mutually, multivariate analysis offers a proper analytical approach (Misra and Uthe, 1987b; Misra *et al.*, 1989c). It is important that the statistician be part of the team designing the study and that he/she also be involved with the quality management at all stages of the study as well as being responsible for the statistical analysis of the data. It must be noted that the proper design of a monitoring programme will require some real data upon which the study can be designed.

2.10 Consult the Experts without Hesitation

Various scientists assisted in the study, particularly during the planning stages, when fish population experts, gear specialists (net characteristics), hydrographers, biochemists, etc., participated as needed to ensure that a minimum number of "surprises" were encountered. Although the present study was simply an investigation of the usefulness of cod as monitors of temporal trends in contaminants, it is obvious that, in most studies, the interest is not solely in changes in contaminant levels in the fish themselves, but rather in the ability of the fish to be used as indicators of changes in contaminant levels in the marine environment. In such studies as this, the identification of trends in contaminant levels in the fish cannot be believed to reflect environmental changes without corroborating evidence, such as that supplied by studies of changing input levels of contaminants and changes in the fish population at large.

3 SAMPLING AND MEASUREMENT OF BIOLOGICAL AND CHEMICAL PARAMETERS

Although quality control studies carried out within chemical contaminant monitoring programmes have mainly concentrated on the determination of contaminant concentrations in a tissue sub-sample or surrogate material, experience (Uthe and Chou, 1988) has shown that large errors can occur at various steps of the analytical procedure, including apparently simple analyses, e.g., determining the fat content of a tissue. It must be emphasized that the overall quality of the study is determined by the poorest quality of any of the many steps of the study. In the Canadian study, the approach to the non-chemical analytical steps was to be as consistent as possible at each step and to employ trained, highly motivated individuals.

3.1 Sampling and Storage on Board the Vessel

Following the dumping of the fish haul on the deck, individual specimens were chosen and the total fish length of each was determined using a standard fish measuring board. Each specimen selected was tagged with a metal-rimmed paper tag attached with a string through the lower jaw, wrapped in a large plastic garbage bag as tightly as possible to prevent freeze-drying, and frozen flat on the right side in the on-board freezer. Other than the incision through the lower jaw for the tag, the fish was not opened in any way, i.e., the animal functionally served as its own container to prevent shipboard contamination. The fish were brought to the laboratory frozen, where they were held frozen for as short a period as possible until tissue dissection.

3.2 Sampling and Storage within the Laboratory

Each fish was allowed to soften under conditions which did not allow any thawing to occur. Length and total body weight were measured, the body cavity opened, the gall bladder checked for integrity, removed, and discarded, and the liver removed intact. Any fish in which the gall bladder had leaked (indicating a degree of post-mortem change) was discarded. Carbon steel knives were used because these had been found not to contaminate the tissues with the contaminants being studied. The liver was placed in a washed, tared, wide-mouthed glass jar, the jar reweighed, and the liver homogenized with either a Polytron homogenizer or a Bronson ultrasonic probe (smaller livers). The jar was sealed with a washed, aluminum foil-lined lid and stored at -40 °C until sub-sampled for analysis. The washing procedure had been designed in terms of precautions applicable to both metal and organic analyses. Using a freshly washed knife, both fillets were removed as rapidly as possible, skinned, and homogenized in a Hobart Silent Cutter using a washed, home-made aluminum spatula to aid mixing. Slow dissection must be avoided due to post-mortem changes, such as "drip" (expression of fluid by thawing flesh). Small fillets were homogenized in a Waring Blender with a glass jar, with an equal weight of glass-distilled water to prevent gel formation. In the case of very large fillets (too large for the capacity of the cutter), each fillet was laid flat, sliced transversely approximately every 5 mm, and every second or third slice taken for homogenization. All equipment was carefully washed between specimens and smaller fish were processed first. Sub-samples of the blend were stored in the same manner as the liver.

3.3 Measurement of Biological and Chemical Variables

Following tissue removal, the otoliths were removed for age determination (by biologists well-experienced in reading otoliths). The net weight of the fish was determined. Studies by Scott *et al.* (1983) had shown that this "net weight" value was closely related to the mass of the musculature as determined by careful dissection and weighing. The fat proportions in the liver and muscle were approximated through the use of the "total extractable solid" figure determined

as a part of the organochlorine analysis. Trace metal and organochlorine analytical procedures are given in Misra and Uthe (1987a). Sub-samples for these determinations were taken after partial thawing and rehomogenization of the stored, frozen samples. A sample of glass-distilled water was "processed" in the same manner as the fish tissue to serve as an operational blank for the analytical chemical measurements. Double time studies did not show any serious effect of homogenization on contaminant concentrations.

4 STATISTICAL ANALYSIS

Data on both chemical concentrations and burdens have been statistically analysed (Misra and Uthe, 1987a). One example of the analysis is given below, i.e., multivariate analysis of covariance (MANCOVA) using length as the covariate, as contrasted to the ICES (ICES, 1987) univariate analysis of covariance (ANCOVA) procedure. However, it must be noted that this example does not consider the problem of inequality of annual slopes and considers only the annual linear trend using the mean length value. If slopes are not parallel, a different value for the annual change characterizes different fish lengths. One way of handling the problem of inequality of annual slopes (and inequality of variances) has been described by Misra *et al.* (1990) for the ANCOVA model.

Concentration data analysed by Misra *et al.* (1988) comprised measurements on each fish of ten contaminant-tissue combinations, designated as Y_i, i = 1, . . ., 10 (after transformation to their common logarithms), for MANCOVA. These were: Y_1 (Zn-M), Y_2 (As-L), Y_3 (Cd-L), Y_4 (Cu-L), Y_5 (Hg-L), Y_6 (Se-L), Y_7 (Zn-L), Y_8 (PCB-L), Y_9 (α-HCH-L), and Y_{10} (HCB-L), where L is liver and M is muscle. Six biological characteristics, including body length (cm), were also measured (Misra and Uthe, 1887a). The "best" combination of covariates has not been established, therefore, Misra *et al.* (1988, 1987a) employed one covariate at a time in their MANCOVAs on Y_i. In the following, we present a part of that MANCOVA for temporal trends where the covariate, X, was log length.

Table 1 gives sample sizes, means, and ranges of X and Y_i for concentration data. There is evidence of linear relationships between the log concentrations and length ($P < 0.05$), showing that a covariance procedure is warranted. Technical details are available in Misra *et al.* (1988, 1987a). Years should, therefore, be compared based on their mean Y_i after adjusting these for variations in the values of the covariate X among individuals. Adjusted year-means, Y_i, are shown in Table 2. Note that these adjusted means would be the same for the analysis of covariance (ANCOVA) and for MANCOVA. The overall MANCOVA null hypothesis, H_o, that years do not differ in their vectors of adjusted means of Y_i, was rejected ($P < 0.001$). This showed that significant linear combinations of groups (years) and variables (Y_i) existed. Following this, an examination of the linear time trend vector of adjusted year-means was one of the analyses done by Misra *et al.* (1988) in the MANCOVA. This was significant ($P < 0.001$). Contributions of individual Y_i to the time trend vector were examined based on 95% simultaneous confidence intervals. Table 3 shows the lower and upper confidence limits for each Y_i. A Y variable would contribute significantly to the linear time trend if its two limits were of the same sign (and thus did not enclose zero). Also, increasing or decreasing trends would be indicated if the two limits are both positive or negative, respectively. Using these guidelines, contaminants with significant contributions to the time trend were identified as follows: (a) with both limits positive: Cd-L (Y_3), Cu-L (Y_4), and Hg-L (Y_5), and (b) with both limits negative: Zn-L (Y_1), PCB-L (Y_8), and HCB-L (Y_{10}). These findings are corroborated by visual examination of increasing and decreasing trends of adjusted year-means of individual contaminants (see Figure 1).

Frequently, analyses of real data sets have shown that more than one covariable needs to be employed. ICES has recognized the need for measuring additional biological covariables (ICES, 1987). Scott *et al.* (1978) and Model 5 of the ICES guidelines introduced a multiple linear regression (MLR) model which employs several (biological) regressor variables to analyse for variations in levels of individual contaminants. Biological covariables, particularly those related to fish size, e.g., age, length, weight, are, however, generally mutually correlated. In Misra *et al.* (1989b), a review is given of problems of multicollinearity which occur when regressor variables are correlated in the MLR and an explanation concerning why the principal component-multiple linear regression (MLR-PC) procedure, which employs principal components of covariables as regressor variables in the MLR, will circumvent the problem of multicollinearity. Misra *et al.* (1989b) also investigated time trends in Canadian Atlantic cod based upon MANCOVA of contaminant concentration and burden data which used principal components of six biological variables as regressors.

5 INTERPRETATION

In all multivariate analyses carried out to date, the results have shown significant linear time trends with respect to certain contaminants. However, significant deviations from the linear trend were also observed, suggesting that longer time studies (more samples taken over a longer interval) are needed to determine the true nature of the trend. Care must be taken in interpreting results in which there are "large" deviations from the linear trend. This situation could arise because the true nature of the trend is non-linear or because there are random fluctuations in the data, in which case, the apparent trend could be spurious (Fryer and Nicholson, 1990)

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Table 1. Mean, Minimum, and Maximum Values for X and Y Variables for Concentration Data.

Year	1977 (n = 34)			1978 (n = 44)			1979 (n = 45)			1985 (n = 35)		
Variable	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.
X_1 Length (cm)	1.615	1.447	1.792	1.687	1.505	1.826	1.684	1.493	1.826	1.672	1.462	1.859
Y_1 Zn-M (μg/kg)	3.624	3.526	3.746	3.615	3.546	3.740	3.669	3.555	3.892	3.556	3.389	3.644
Y_2 As-L (mg/kg)	0.291	0.076	0.599	0.243	-0.004	0.650	0.378	0.097	0.858	0.349	-0.032	0.728
Y_3 Cd-L (μg/kg)	1.778	1.342	2.423	2.179	1.763	2.746	1.844	1.415	2.412	2.396	2.093	2.881
Y_4 Cu-L (mg/kg)	0.602	0.255	1.104	0.619	0.057	0.978	0.806	0.037	1.438	0.846	0.598	1.208
Y_5 Hg-L (μg/kg)	1.358	0.903	1.690	1.426	1.000	2.000	1.325	1.000	1.914	1.781	1.447	2.117
Y_6 Se-L (μg/kg)	2.915	2.763	3.117	3.022	2.699	3.305	3.129	2.724	3.597	2.973	2.432	3.199
Y_7 Zn-L (mg/kg)	1.185	1.013	1.365	1.182	0.898	1.387	1.265	1.014	1.511	1.224	0.969	1.452
Y_8 PCB-L (μg/kg)	0.274	0.004	0.719	0.463	0.124	0.808	0.424	0.143	0.872	0.162	-0.194	0.813
Y_9 α-HCH-L (μg/kg)	1.865	1.491	2.076	1.815	1.342	1.987	1.809	1.255	2.025	1.792	1.431	2.013
Y_{10} HCB-L (μg/kg)	1.631	1.491	2.067	1.658	0.845	1.903	1.624	1.114	1.826	1.330	0.845	1.556

Table 2. Adjusted year-means of Y variables.

	Year			
Variable	1977	1978	1979	1985
Y_1	3.700	3.695	3.748	3.636
Y_2	-0.269	-0.343	-0.206	-0.231
Y_3	0.545	0.891	0.558	1.119
Y_4	-0.069	-0.081	0.107	0.152
Y_5	-0.133	-0.131	-0.229	0.237
Y_6	2.172	2.246	2.355	2.203
Y_7	0.991	0.978	1.062	1.023
Y_8	-1.840	-1.747	-1.781	-2.027
Y_9	2.513	2.491	2.484	2.462
Y_{10}	1.550	1.574	1.540	1.247

Table 3. Lower and upper confidence limits for individual contaminants in the time trend contrast based on 95% simultaneous confidence intervals.

Variable	Lower Limit	Upper Limit
Y_1	-2.5429	-0.3829
Y_2	-1.7934	4.0283
Y_3	5.4114	13.7729
Y_4	0.3253	8.0500
Y_5	4.1077	11.9895
Y_6	-3.2559	2.4278
Y_7	-1.3144	2.4038
Y_8	-7.5194	-2.0105
Y_9	-3.2309	1.5976
Y_{10}	-9.7174	-3.2946