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Section 04—Regulatory
EEDP-04-18 through EEDP-04-26

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Environmental Effects of Dredging Technical Notes



Dioxin in Sediments: Application of Toxic Equivalents Based on International Toxicity Equivalency Factors to Regulation of Dredged Material

Purpose

This technical note explains the origin and meaning of the dioxin toxic equivalent (TEQ) concept, reviews the application of TEQs to dredged sediment evaluations, examines the underlying assumptions of the application, considers appropriate and inappropriate usage, and discusses a possible alternative to the analytical chemistry-based calculation of TEQs.

Background

A dioxin TEQ expresses the toxicity of a mixture of related compounds in a sample as though the sample contained an equivalent amount of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), thought to be the most toxic environmental contaminant. TEQs have been used in risk assessment in some of the European states for several years.

The method was standardized in 1988 using the International Toxicity Equivalency Factors (I-TEFs) proposed by the NATO Committee on the Challenges of Modern Society (CCMS) Pilot Study on International Information Exchange on Dioxin and Related Compounds (CCMS 1988a,b). The I-TEF method has now been adopted by Canada and the United States, as well as the Netherlands, Great Britain, and the Nordic countries.

Although intended as a procedure for human health risk assessment, TEQs have recently been extended in use to the regulation of open-water disposal of dredged sediments. Some regional offices of the U.S. Environmental Protection Agency (USEPA) and several State resource agencies have either implemented the use of TEQs or propose to require their use in environmental regulation.

In concept, the I-TEF method for calculation of dioxin TEQs can be applied whenever a sample contains measurable amounts of any of the polychlorinated dibenzo-*p*-dioxin (PCDD) or polychlorinated dibenzofuran (PCDF) congeners for which toxic equivalent factors (TEFs) have been assigned. The toxicity of these compounds is thought to be additive, and summation of TEFs is considered to express the potential toxicity of the sample as though it contained an equivalent amount of 2,3,7,8-TCDD.

It is not necessary that 2,3,7,8-TCDD itself be detected in the sample, and polychlorinated biphenyls (PCBs) and other chemicals structurally related to the PCDDs and PCDFs are not included in the I-TEF calculation. The calculation of TEQs using TEFs involves highly expensive trace chemical analysis procedures and has other drawbacks as well as significant strengths.

Additional Information

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Introduction

Polychlorinated dibenzo-*p*-dioxins, especially 2,3,7,8-TCDD, are among the most toxic and persistent of environmental contaminants. These and the structurally similar PCDFs, the PCBs, and other groups of polyhalogenated aromatic hydrocarbons (PHHs) are associated with genotoxic and cytotoxic effects, as well as body weight loss, reproductive impairment, acute lethality, chloracne, liver damage, edema, and other toxicities (Greig 1979, Kociba and Cabey 1985, Kociba and others 1978, Safe 1987). Much concern has arisen in recent years over the widespread occurrence and potential for toxicity of these chemicals in the aquatic environment, including sediments slated for dredging and disposal.

Most dioxin research to date has focused on 2,3,7,8-TCDD. Nevertheless, there are thousands of other PHH compounds, including 75 PCDD congeners and 135 PCDF congeners, and it is appealing to try to understand the potential toxicity of some of these related compounds in terms of the more familiar (and most toxic) 2,3,7,8-TCDD. Thus, dioxin "toxic equivalents" have been formulated in an attempt to express the combined toxicity of a mixture of PHH in a sample as though the sample contained an equivalent amount of 2,3,7,8-TCDD alone.

The rationale for TEQs is the fact that substances with molecular structures similar to 2,3,7,8-TCDD (that is, those that are isosteric) exhibit the same kind of toxicities, differing mainly in potency of the effect. This phenomenon proceeds from the fact that reversible binding to an intracellular receptor protein, the *Ah* receptor, is the initial event in the series of steps that lead to dioxin-type toxicities. Binding to the *Ah* receptor requires certain molecular structural

characteristics shared by 2,3,7,8-TCDD and its PHH isosteres. A PHH can be assigned a TEF expressing its toxicity as a fraction of 2,3,7,8-TCDD toxicity. The product of the concentration of a PHH compound and its TEF normalizes the toxicity of that compound in a sample to an equivalent amount of 2,3,7,8-TCDD. Summation of the products of TEF and PHH concentrations in a sample yields a TEQ. The TEQ can then be treated as though it were the concentration of 2,3,7,8-TCDD in the sample for purposes of risk assessment.

This technical note describes the use of TEQs in regulatory decision-making processes involving dioxin-containing dredged sediments. Shortcomings in the present use of TEQ methodology are described and supported by examination of recent cases where TEQs have been used in regulatory decisions. An alternative approach based on bioassay-derived TEQs shows promise in overcoming many of the problems associated with TEQs as currently derived from chemical analysis.

TEQs in Aquatic Environmental Assessments

Dioxin TEQs were standardized in 1988 using International Toxicity Equivalency Factors (I-TEFs) (Table 1). The derivation of I-TEFs was based on several criteria; however, a single long-term carcinogenicity study on rats (Kociba and Cabey 1985, Kociba and others 1978) was given the highest priority (CCMS 1988a,b; Kutz and others 1990; Safe 1990). As such, I-TEFs do not reflect the large variability observed when the potency of individual PHHs is compared with the potency of 2,3,7,8-TCDD using specific responses in different organisms. For example, there is a nine hundred-fold difference for one coplanar PCB congener in the TEF calculated for aryl hydrocarbon hydroxylase (AHH) induction in chick embryo and in intact rat (Table 2).

I-TEFs were never developed with ecological protection in mind. Instead, the I-TEFs represent a synthesis reached by a committee of experts using ranked criteria in which potential carcinogenicity in humans was given first priority. All data used in the derivation of I-TEFs were obtained from mammalian (primarily rodent) studies. Thus, if I-TEFs are used to calculate TEQs in evaluations of dioxin-contaminated sediment effects on aquatic biota, there must be an implicit assumption of a parallel between potency for human carcinogenicity and toxic effect in submammalian species.

The research supporting this assumption remains to be done. In the interim, the most appropriate application of I-TEF-based TEQs in environmental assessments is in terms of risk to human consumers of contaminated fish and shellfish. If used in this context, I-TEFs appear to represent the best approximation presently available for the interpretation of analytical chemical data in toxicological terms.

I-TEFs have been agreed upon for 17 PCDD and PCDF congeners containing the chlorine 2,3,7,8-substitution pattern. Not included are the PCBs and other structurally related PHHs. Some of these compounds, particularly the

Table 1. International Toxicity Equivalency Factors

<u>PCDD Congener</u>	<u>I-TEF</u>	<u>PCDF Congener</u>	<u>I-TEF</u>
2,3,7,8-TCDD	1	2,3,7,8-TCDF	0.1
1,2,3,7,8-PeCDD	0.5	2,3,4,7,8-PeCDF	0.5
		1,2,3,7,8-PeCDF	0.05
1,2,3,4,7,8-HxCDD	0.1	1,2,3,4,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDD	0.1	1,2,3,7,8,9-HxCDF	0.1
1,2,3,6,7,8-HxCDD	0.1	1,2,3,6,7,8-HxCDF	0.1
		2,3,4,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-HpCDD	0.01	1,2,3,4,6,7,8-HpCDF	0.01
		1,2,3,4,7,8,9-HpCDF	0.01
OCDD	0.001	OCDF	0.001

Table 2. Toxic Equivalent Factors Calculated for 3,3',4,4'-Tetrachlorobiphenyl for Several Responses and Species¹

<u>Response</u>	<u>TEF</u>
Body weight loss (rat)	<0.0001
Thymic atrophy (rat)	<0.0002
Thymic lymphoid development (mouse)	0.00067
AHH induction, in vitro (H4IIE cell line)	0.001 to 0.002
AHH induction, in vivo (rat)	0.00001
AHH induction, in vitro (chick embryo hepatocytes)	0.009
Receptor binding	0.0023

¹ From data presented in Table 15 of Safe (1990).

coplanar PCBs, may pose a greater threat to both wildlife and humans than do the dioxins and furans (Dewailly and others 1991; Niimi and Oliver 1989; Tanabe and others 1987a,b).

Safe (1990) proposed an expansion of the I-TEF list to include coplanar polychlorinated and polybrominated biphenyls, along with brominated and bromo/chloro dibenzo-*p*-dioxins and dibenzofurans. Such an expansion appears appropriate for the coplanar PCBs since these chemicals are abundant and are apparently becoming enriched rather than disappearing from the environment (Tillet and others 1992).

Because I-TEFs are summed to obtain a TEQ, additivity of toxic effect of the individual PCDD and PCDF congeners is assumed, and possible synergism or antagonism is ignored. In fact, antagonistic effects among PHH congeners in a mixture have been demonstrated in a number of cases. The PCB mixture

Aroclor 1254, other Aroclor mixtures, and specific individual PCB, PCDD, and PCDF congeners have all been shown to antagonize the toxic effects of 2,3,7,8-TCDD in mammalian studies (Astroff, Romkes, and Safe 1989; Bannister and others 1987; Davis and Safe 1990; Haake and others 1987; Prokipcak and others 1990; Waern et al. 1989, 1990).

The current method of calculating TEQs from I-TEFs and analytical chemistry thus has several shortcomings that limit the utility of the method for environmental regulatory evaluations, not the least of which is high cost. In fact, the I-TEF method was intended by its developers to be only an interim approach that should be replaced, as soon as practicable, by a more definitive bioassay for the determination of TEQs (Barnes 1991, Kutz and others 1990).

Dredged Sediment Evaluations Using TEQs

I-TEF-based TEQs have recently been required in some environmental assessments. The State of Oregon, for example, has promulgated recommendations on the use of TEQs in environmental regulations (Oregon Department of Environmental Quality 1990). The USEPA has adopted TEQs in risk assessment and in rule making, but has not been consistent in their application. For example, in a recent regulatory decision, Region 10 of the USEPA, in conjunction with the Oregon Department of Environmental Quality, the Washington Department of Ecology, and the Idaho Department of Environmental Quality, set a total maximum daily loading value of 6 mg/day 2,3,7,8-TCDD for the Columbia River Basin based solely on water quality criteria for 2,3,7,8-TCDD, not on TEQs.

On the other hand, several U.S. Army Corps of Engineers (USACE) elements have recently been asked to use TEQs rather than actual concentrations of 2,3,7,8-TCDD in decision making for Federal navigation projects. One such case involved a risk assessment performed by the USACE District, Seattle, in conjunction with maintenance dredging of the Federal Channel at Gray's Harbor, Washington (USACE 1991).

Several tiers of the dredged sediment evaluation tiered testing protocol outlined in the "Green Book" (USEPA/USACE 1991) were performed concurrently to save time. 2,3,7,8-TCDD was detected in only 3 of 17 sediments, at concentrations ranging from 1.5 to 3.9 parts per trillion (pptr). 2,3,7,8-substituted PCDDs were present in some sediment samples, but at such low concentrations that there was no "reason to believe," in a Tier II evaluation of the sediments, that dioxin would be bioaccumulated to detectable levels. All sediment toxicity tests were negative and bioaccumulation tests were inconclusive; thus, there were no Tier III exceedances.

Nevertheless, the District was compelled by the USEPA and state agencies to perform a TEQ-based human health risk analysis on the project sediments. The risk analysis was performed with data generated by assuming concentrations to be equal to one half the detection limit since most samples contained

no detectable dioxins or furans. The outcome of the risk assessment was no incremental human health risk attributable to these compounds.

In another case, the USACE District, Walla Walla, was delayed in 1991 from performing a previously approved maintenance dredging project in the upper Snake River when the "104 Mill Survey" identified a nearby industrial source of dioxin. This delay was resolved by an agreement between the District and USEPA Region 10 to sample the sediments slated for dredging for selected dioxin and furan congeners.

Because the cost of dioxin determinations is so high, the District proposed a plan whereby dioxin would be analyzed only in sediments with the highest total organic carbon (TOC) content (those in which dioxin could be expected to be found, if present). Sediments were collected throughout the project area, and TOC was determined in all samples. The sediment samples were archived until initial dioxin testing of the highest TOC samples was complete. If dioxins were found in the high-TOC samples, the next highest TOC samples would then be analyzed. The analytical results would be used to calculate TEQs.

In a third case involving TEQs, the National Oceanographic and Atmospheric Administration Natural Resources Trustees recently presented the USACE District, Charleston, with concerns regarding dioxin contamination in Winyah Bay, South Carolina. As a result of the "104 Mill Survey," the South Carolina Department of Health and Environmental Control (SCDHEC) sampled organisms and sediments throughout Winyah Bay. They found a few organisms with elevated levels of dioxin TEQs, and 5 of 11 sediment samples had dioxin TEQ levels above 2 pptr.

In January and February 1989, 22 stations were sampled for organisms. Of these samples, 14 exceeded 1 pptr TEQ, and 3 had TEQs exceeding the 25-pptr U.S. Food and Drug Administration (FDA) limit for 2,3,7,8-TCDD in edible fish portions. In August and September 1989, SCDHEC sampled 51 organisms for dioxins. Of these, 24 had TEQs exceeding the 1-pptr detection limit routinely obtained for dioxin in tissue samples, and one exceeded the 25-pptr FDA limit (unpublished data, SCDHEC). Congeners analyzed in the tissue samples were the 17 I-TEFs listed in Table 1; of these, the most frequently occurring were 2,3,7,8-TCDD, OCDD, and 2,3,7,8-TCDF. The Charleston District is evaluating Federal project sediments for three reaches of Winyah Bay using guidance published in the "Green Book" (USEPA/USACE 1991).

Regulatory evaluations of dioxin-containing sediments in the New York-New Jersey Harbor area have been based on the bioaccumulation of 2,3,7,8-TCDD, rather than on TEQs. Bioaccumulation testing using the polychaete *Nereis virens* is performed if dredging project sediments exceed 1 pptr 2,3,7,8-TCDD.

In 1992, the USACE District, New York, proposed guidelines for evaluating dioxin bioaccumulation data (personal communication, John Tavolaro, New York District). If bioaccumulation levels in worms exposed to the dredged sediment were significantly greater (95 percent confidence level) than

bioaccumulation levels in worms exposed to reference sediment, the restrictions described below would apply.

For bioaccumulation of at least 1 pptr 2,3,7,8-TCDD and less than 10 pptr in worms exposed to the dredged sediment, ocean disposal would be allowed and expeditious capping would be required (within 2 weeks, 2 to 1 ratio of cap to capped material). For bioaccumulation of at least 10 pptr and less than 25 pptr, expeditious capping would be required (within 10 days, at least 2 to 1 ratio of cap to capped material), and special measures (such as onboard inspectors) would be taken to ensure that the material was accurately placed and capped. For bioaccumulation of 25 pptr and above, ocean disposal would not be allowed. These protocols have been accepted by the USEPA Region 2 and are to be reassessed within 18 months after completion of the first dredging project involving dioxin evaluation.

As the above examples demonstrate, the regulation of dioxin-containing sediments is far from standardized on a national basis. More research into the relationship between sediment levels and toxicity is certainly required.

Strengths and Weaknesses of I-TEF-based TEQs

Dioxin TEQs are beginning to play a role in environmental evaluations, including regulatory decision making with regard to dredged sediments. Although the calculation of TEQs has been standardized using I-TEFs, their application by state and federal regulatory agencies is by no means consistent. The strengths and weaknesses of I-TEF-based TEQs in environmental evaluations can be summarized as shown below.

Strengths

- Able to recognize the contribution to toxicity of compounds other than 2,3,7,8-TCDD.
- Express the toxic potential of a sample in terms of a single numerical value.
- Provide a means of relating chemical analytical data to biological effect.
- Limits of detection are those of the chemical analysis, presently on the order of 100 to 200 parts per quadrillion for individual congeners.
- When applied to sediment analyses, can be used to determine the necessity for Tier III or Tier IV biological testing.

Weaknesses

- Restricted to PCDDs and PCDFs; not included are PCBs or other structurally related PHHs, some of which are much more abundant in the environment and thus may have greater toxic potential than the dioxins and furans.
- Necessitate highly expensive trace chemical analysis.

- Account for only additivity of toxic effect of the congeners in a mixture, whereas antagonistic effects have also been demonstrated.
- Do not account for the large (several orders of magnitude) species- and response-dependent variability in empirical toxic equivalent factors.
- Are biased toward human health protection and may not accurately assess the real toxicity of dredged material to aquatic biota.
- When applied to sediment data alone, do not address bioavailability, that is, the dose actually delivered to the animal.

Biological Alternatives

TEQs provide a way to express the toxicity of complex mixtures of environmental contaminants that is highly appealing for its simplicity. Basing TEQs on an integrative bioassay rather than on trace chemical analysis would overcome most of the weaknesses mentioned above while retaining most of the strengths, including the simplicity of a single 2,3,7,8-TCDD-equivalent number. One such bioassay is the H4IIE in vitro bioassay, which uses the rat hepatoma H4IIE cell line (Bradlaw and Casterline 1979). This bioassay integrates the additive and antagonistic effects of a mixture into a numerical result (the TEQ) at a cost per sample of 10 to 20 times less than trace chemical analysis.

The H4IIE assay makes use of the fact that toxic potency of dioxin-like compounds correlates strongly with the potency of these compounds to cause induction of certain xenobiotic-metabolizing enzymes. Two of these marker enzymes, ethoxyresorufin-O-deethylase (EROD) and AHH can be measured using highly sensitive fluorescence spectrophotometry, approaching the resolution of gas chromatography/electron capture detection (GC/ECD) at much lower cost. The potency of a mixture of dioxin-like compounds can be compared with the potency of a pure 2,3,7,8-TCDD standard for the induction of AHH and/or EROD using the H4IIE cell line, and the result can be expressed as a TEQ.

The H4IIE cell line has been used to measure TEQs in fish extracts (Casterline and others 1983; Zacharewski, Safe, and Safe 1989) and in the eggs of fish-eating waterbirds (Tillet, Ankley, and Geisy 1989; Tillet and others 1991, 1992). Recently, the procedure was applied to sediments (personal communication, John P. Geisy, Michigan State University) and is now being investigated by the U.S. Army Engineer Waterways Experiment Station as a new procedure for dredged sediment evaluation.

The H4IIE cell line has been used since 1961 (Casterline and others 1983); however, it has only recently begun to find its way into widespread environmental applications and may soon be eclipsed by simpler and more sensitive procedures. Recently, recombinant methods were used to insert dioxin-responsive segments of human genes into a plasmid containing the firefly luciferase gene. In the presence of dioxin or related compounds, the gene responds by expressing luciferase, which can be measured quantitatively with a luminometer (Postlind and others 1992). The method is similar to the H4IIE assay, but is simpler and may prove to be even more sensitive. It appears likely that

advances, such as this, in molecular biology will result in the development of more specific, sensitive, rapid, and less expensive alternatives to analytical chemistry for measuring TEQs.

Conclusions

Use of the I-TEFs to calculate a dioxin TEQ in an environmental sample is an attractive and simple means of relating chemical concentration data to the potential for a toxic effect. Although developed for use in human risk assessment, the concept and practice have been extended to ecological evaluations, including evaluations of dredged sediments intended for open-water disposal. Despite numerous limitations when applied to ecological evaluations of contaminants in dredged sediments, the use of I-TEF-based TEQs provides a means of obtaining toxicologically relevant information from sediment chemistry. Biological methods now under development have the potential of reducing or eliminating many of the problems inherent in the use of I-TEFs.

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Environmental Effects of Dredging Technical Notes



The Use of Population Modeling to Interpret Chronic Sublethal Sediment Bioassays

Purpose

This technical note provides a brief introduction to population modeling and describes the application and utility of such techniques for dredged material bioassays. The use of population modeling as a source of interpretive guidance for chronic sublethal dredged material bioassays is emphasized.

Background

Current laws and regulations governing the discharge of dredged material stress the importance of assessing the chronic (long-term) sublethal effects of dredging operations. Regulations implementing section 103 of the Marine Protection, Research and Sanctuaries Act (PL 92-532) state that, "Materials shall be deemed environmentally acceptable for ocean dumping only when . . . no significant undesirable effects will occur due either to chronic toxicity or to bioaccumulation . . ." Similar language is used in regulations implementing section 404(b)(1) of the Clean Water Act (PL 92-500) which reads: "The permitting authority shall determine in writing the potential short-term or long-term effects of a proposed discharge of dredged or fill material on the physical, chemical, and biological components of the aquatic environment . . ." It also stipulates that tests "may be required to provide information on the effect of the discharge material on communities or populations of organisms."

Populations and their aggregations, communities, represent the level of biological organization of greatest interest to society in general as well as to regulators (Figure 1). This interest is expressed as concern for the effects of contaminants on maintaining viable populations of commercially important species, such as oysters or striped bass, as well as other members of aquatic and marine systems. Because of the complexity inherent at the population/community level of biological organization, predicting contaminant effects at

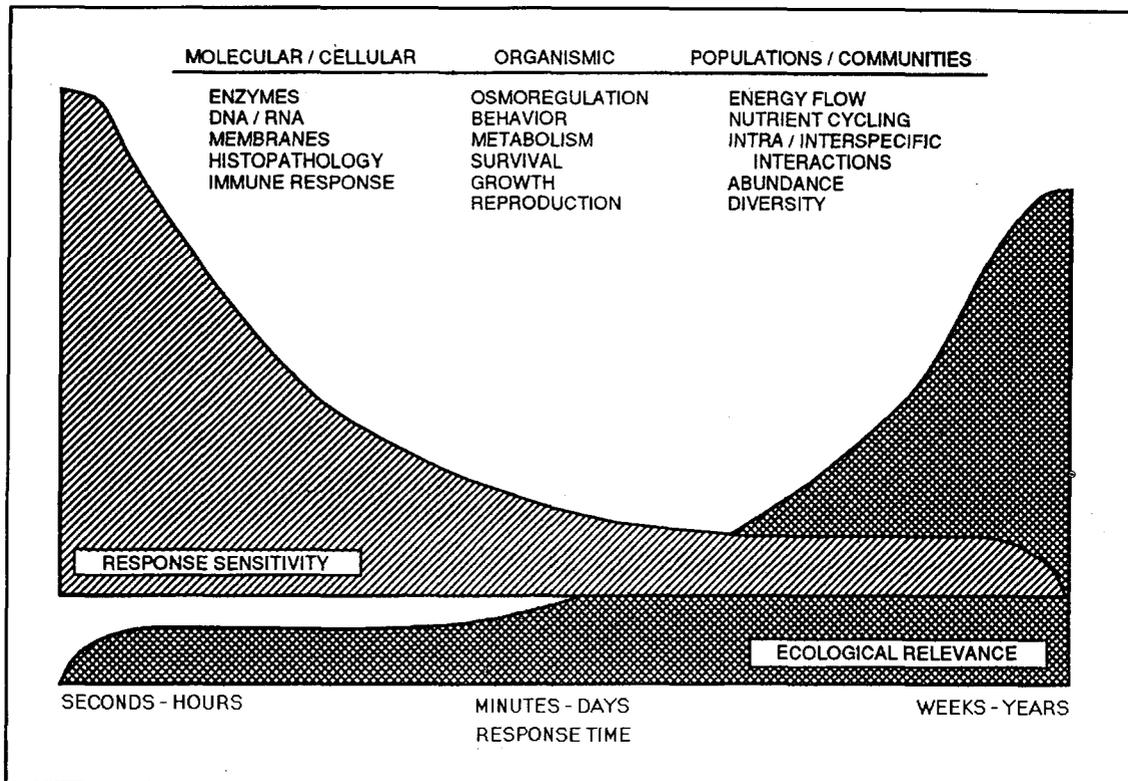


Figure 1. Levels of biological organization. Three levels of biological organization associated with chronic sublethal bioassays are listed with examples of relevant level-specific processes and time scales. Bioassays at the molecular/cellular level are highly sensitive and produce results in short periods of time, but tests conducted at this level lack ecological relevance. Conversely, tests focused on populations/communities in the field have the greatest ecological relevance, but require long periods of time to perform and produce results which are difficult to interpret. Consequently, bioassays performed at the organismic level represent the optimum tradeoff between response sensitivity and ecological relevance.

this level is difficult. Consequently, the focus of dredged material testing has been on lower levels of organization where responses to contaminants are more easily recognized and understood. However, the effective use of bioassays conducted at lower levels of biological organization, that is, the molecular/cellular and organismic levels, requires the establishment of a meaningful link between results of these tests and population viability or health. How can bioassays conducted at these lower levels of organization be used to predict effects at the population/community level? Population modeling techniques provide the only mechanism for establishing this link.

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Interpretive Guidance for Chronic Sublethal Sediment Bioassays

A number of federal, academic, and private laboratories in the United States are currently developing chronic sublethal sediment bioassays for the evaluation of dredged material (Dillon, Gibson, and Moore 1990). Most chronic sublethal tests under development use either polychaete or amphipod species because these animals are amenable to such testing. Potential chronic sublethal test endpoints include growth, reproduction, behavior, and physiological measures of metabolic rate.

Interpretive guidance, the establishment of a link between test endpoints and ecological effects, is a necessary component of a fully developed chronic sublethal sediment bioassay (Dillon, Gibson, and Moore 1990 and Dillon 1992, 1993). The two most commonly used sublethal endpoints, growth and reproduction, provide sensitive measures of animal stress during chronic exposures to contaminated sediments (Dillon, Moore, and Gibson 1993, McGee, Schlekat, and Reinharz 1993, and Moore, Dillon, and Suedel 1991). However, the ecological meaning of growth and reproductive responses is difficult to quantify. Since current regulations are focused on protecting populations of organisms, one might ask "What would a 10 percent reduction in growth or reproduction in animals exposed to sediment during a bioassay mean for populations in the field?" Without well developed interpretive guidance, this question is impossible to answer. Population modeling represents an efficient and powerful technique for providing the necessary interpretive guidance for chronic sublethal sediment bioassays (Dillon, Gibson, and Moore 1990, Gentile and others 1982, and Pesch, Munns, and Gutjahr-Gobell 1991).

Data Requirements for Population Modeling

The construction of a population or demographic model generally requires the collection of data on survivorship, growth, and reproduction over the entire life span of the organism of concern. Chronic sublethal sediment bioassays are particularly amenable to using demographic models since these bioassays are commonly run over a major portion of the life cycle of test organisms and the endpoint data collected (survivorship, growth, and reproduction) constitute the necessary elements of a demographic model.

In laboratory settings, demographic data are collected by raising a number of individuals from birth through death under controlled conditions and collecting information on survivorship, growth, and reproduction by monitoring individuals at regular intervals (for example, daily or weekly). This procedure, though not practical for long-lived species, can be effectively applied to most species used in chronic sublethal testing since life cycles in these species are typically short (days to weeks). Two common approaches to population modeling use life table analysis and matrix population modeling.

Life Tables and Calculation of the Intrinsic Rate of Natural Increase (r)

The dynamics and structure of populations can be described by such vital rates as birth, growth, development, and mortality (Caswell 1989). Early attempts at describing populations in these terms resulted in the development of life tables (Pearl 1928, Bodenheimer 1938, and Deevey 1947). In their simplest form, life tables contain age-specific information on survivorship (l_x) and reproduction (m_x) (Table 1). A number of parameters which describe aspects of population structure and dynamics can be calculated from l_x and m_x , including r , the intrinsic rate of natural increase. Using Euler's (1970) equation:

$$\sum_{x=0}^{\infty} e^{-rx} l_x m_x = 1 \quad (1)$$

where x is the age class, and r can be calculated using data from a life table. When the per capita birth rate (b) of a population exceeds the per capita death rate (d), r , which represents a per capita growth rate, is positive ($r = b - d$), and the population is growing. When the death rate exceeds the birth rate, r is negative, indicating that the population is declining. Such summary values as r serve to integrate age-specific information on survivorship and reproduction into a single, standard value that encapsulates the status of a population. In fact, by using the equation

$$N_t = N_0 e^{rt} \quad (2)$$

Age (x)	Survivorship (l_x)	Fecundity (m_x)
0	1.0	0.0
1	0.8	0.0
2	0.7	0.2
3	0.6	0.5
4	0.4	1.0
5	0.2	0.3
6	0.0	0.0

Note: Survivorship (l_x) represents the proportion of individuals present at age 0 (newborns) that are alive at age x ; l_x can be viewed as the probability that a newborn will be alive at age x . Fecundity (m_x) represents the average number of offspring produced by an individual of age x during that age period.

population size at any time (N_t) can be calculated by knowing the population's initial size (N_0), r , and the amount of time transpired (t). However, Equation 2 will only accurately predict population size at time t (N_t) if population growth can be described in terms of an exponential growth curve (Figure 2). Other model formulations of population growth have been developed in recognition of the fact that populations in nature rarely, if ever, experience exponential growth (for example, due to limiting resources) (Pielou 1977).

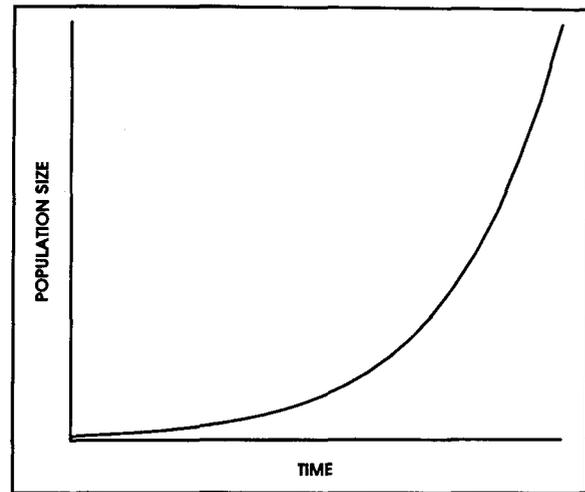


Figure 2. Exponential curve describing population growth over time using Equation 2

Matrix Population Models and Calculation of the Finite Rate of Increase (λ)

By use of matrix algebra and population projection matrices, which contain life table data in a slightly different form, matrix population models provide relatively convenient methods for deriving useful descriptive statistics for population dynamics and structure (Figure 3). Matrix population models were

$$\begin{array}{c}
 \mathbf{n}_{(t+1)} \\
 \left[\begin{array}{c} n_1 \\ n_2 \\ n_3 \\ n_4 \\ \vdots \\ n_s \end{array} \right]_{(t+1)}
 \end{array}
 =
 \begin{array}{c}
 \mathbf{A} \\
 \left[\begin{array}{ccccccc} F_1 & F_2 & F_3 & F_4 & \dots & F_s \\ P_1 & 0 & 0 & 0 & \dots & 0 \\ 0 & P_2 & 0 & 0 & \dots & 0 \\ 0 & 0 & P_3 & 0 & \dots & 0 \\ \vdots & \ddots & \ddots & \ddots & \dots & \vdots \\ 0 & 0 & 0 & \dots & P_{s-1} & 0 \end{array} \right]
 \end{array}
 \begin{array}{c}
 \mathbf{n}_{(t)} \\
 \left[\begin{array}{c} n_1 \\ n_2 \\ n_3 \\ n_4 \\ \vdots \\ n_s \end{array} \right]_{(t)}
 \end{array}$$

Figure 3. Population projection. This entire expression may be more simply expressed as $\mathbf{n}_{(t+1)} = \mathbf{A}\mathbf{n}_{(t)}$. \mathbf{A} is called a population projection matrix; in this age-classified case this matrix is also known as a Leslie matrix. The first row of \mathbf{A} contains age-specific fecundities (F_s) and the subdiagonal contains age-specific survival probabilities (P_s). The two single column matrices (or vectors) contain the number (n) of individuals in each age class at time t and one time step later ($t+1$). \mathbf{A} is referred to as a projection matrix because when it is multiplied by $\mathbf{n}_{(t)}$ the resulting vector ($\mathbf{n}_{(t+1)}$) contains the number of individuals in each age class after one time step of growth (that is, population size has been projected one time step into the future).

developed independently during the 1940s by Bernardelli (1941), Lewis (1942), and Leslie (1945), but were not in common use by ecologists prior to the 1970s (Caswell 1989).

One descriptive statistic produced by matrix population techniques is the finite rate of increase (λ), which summarizes the effect of a population projection matrix. In the case of λ , values greater than 1 mean a population is growing and values less than 1 mean a population is declining. The use of λ as a measure of population growth rate is indicated in the following equation:

$$N_t = N_0 \lambda \quad (3)$$

A population growing at a rate of $\lambda = 1.2$ /week would be increasing by 20 percent per week.

One attractive feature of matrix population models is their flexibility. This flexibility is particularly beneficial when the organism of interest has a complex life cycle where it is helpful to classify organisms according to factors other than age, for example, size or developmental stage (Caswell 1989). Complex life cycles are common among organisms used in sediment bioassays.

Using r or λ : Their Relationship and Assumptions

The life table and matrix population modeling techniques described above for producing the summary statistics r and λ actually produce equivalent results. In fact, the relationship between λ and r is related by the equations:

$$r = \log_e \lambda \quad (4)$$

or

$$\lambda = e^r \quad (5)$$

Even though the two techniques produce equivalent results, there are advantages to using matrix population models over the life table technique described. For example, the mathematics of matrix population models is more convenient than life table techniques (Caswell 1989). Other advantages of matrix population models include their flexibility as well as the fact that the meaning of λ can be more easily understood and communicated. (Equation 3 is relatively more simple than Equation 2.)

The two most important assumptions of the techniques described above involve the constancy of environmental conditions and the equilibrial status of populations. The summary statistics r and λ are based on calculations using data on survivorship and reproduction collected under a specific set of environmental conditions (usually laboratory conditions). If conditions in the field

differ from those in the laboratory in such a way that survivorship and reproduction are affected, then the summary statistics (for example, λ) may not accurately reflect population growth in the field. Additionally, the summary statistics accurately describe population growth only after the population has reached a stable age distribution; that is, the population has reached an equilibrium state in which the proportion of individuals in each age class remains constant through time. This equilibrium state is rarely if ever reached in natural populations.

Even with these limiting assumptions, population models represent a powerful way of projecting effects on populations using data collected on individual organisms. The limiting assumptions mentioned above are at least partially overcome by stochastic demographic modeling (Ferson 1991 and Burgman, Ferson, and Akcakaya 1993), a technique particularly suited for application in ecotoxicology and dredged material testing.

Application to Bioassays

Beginning with Marshall (1962), a number of studies have made use of demographic concepts and models in ecotoxicology. A variety of organisms have been used in life table response experiments (Caswell 1989) to estimate the population-level consequences of contaminants including cladocerans (Chandini 1991 and Wong and Wong 1990) and other crustaceans (Gentile and others 1982), polychaetes (Pesch, Munns, and Gutjahr-Gobell 1991), oligochaetes (Niederlehner and others 1984), nematodes (Vrannken and Heip 1986), gastrotrichs (Hummon 1974), and rotifers (Rao and Sarma 1986).

The summary statistics produced by demographic models are useful descriptors of population health or viability. For example, populations experiencing positive growth could be described as healthy, while populations with a λ less than 1 would be at risk of extinction if the environmental conditions producing the population decline persist. Such a measure of population health (λ) effectively integrates the effects contaminants have on organism survivorship, growth, and reproduction. In designing a dredged material bioassay, one might ask the question "Does population growth (λ) differ in a biologically significant way in animals exposed to dredged material compared to reference sediments?"

Answering the preceding question is the foundation of interpretive guidance for chronic sublethal bioassays. If the results of a dredged material bioassay indicate a 10 percent reduction in growth in animals exposed to dredged material compared to reference site sediment, but the λ 's for dredged material and reference exposed animals are essentially identical, one would expect the 10 percent reduction in growth to have little or no effect on population health. Used in this fashion demographic models and their summary statistics would suggest biologically reasonable criteria for judging the toxicity of sediments. If population growth is only affected when individual growth is reduced by

30 percent, then 30 percent represents a reasonable mark for judging a sediment as contaminated.

A Hypothetical Example

Figure 4 contains hypothetical results for a series of chronic sublethal sediment bioassays where data were also collected to provide interpretive guidance in the form of population growth rate (λ). The vertical axis lists the population growth rate of test animals exposed to the sediments listed on the horizontal axis. Sediment A is from a reference station, while sediments B through F are project sediments.

The population growth rate for test animals exposed to project sediment B is the same as for animals exposed to the reference sediment. Population growth rates of animals exposed to project sediments C and D were reduced in comparison to the reference sediment, but were still greater than 1. Since populations exposed to project sediments C and D were experiencing positive growth, they may be considered healthy. Population growth is further reduced in project

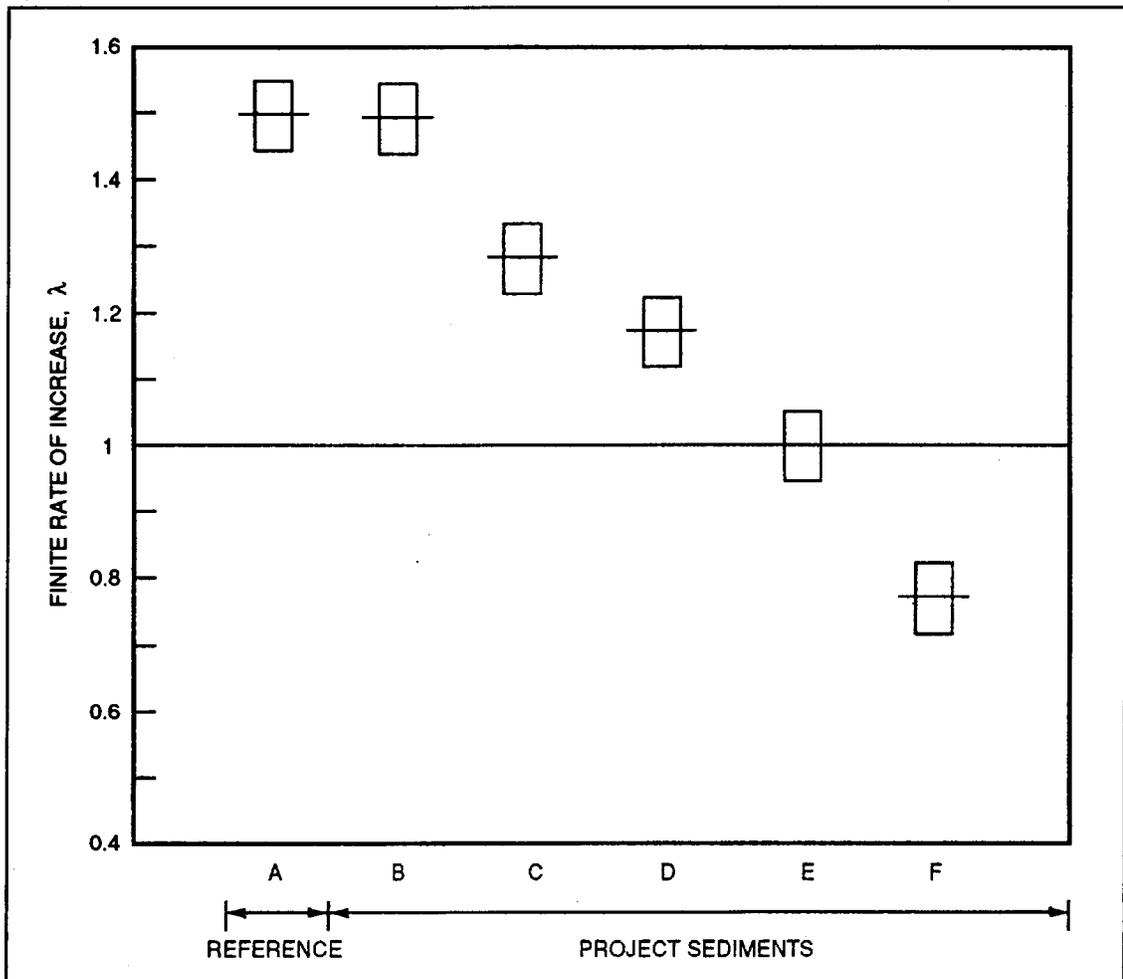


Figure 4. Results of a hypothetical series of dredged material bioassays

sediment E; in fact, λ equals 1, indicating that the population is neither growing or declining but maintaining a constant size. It would be reasonable to declare sediments resulting in a λ of less than 1 to be contaminated since a population growing at such a rate would go extinct given sufficient time (project sediment F). Used in this fashion, population modeling can provide meaningful interpretive guidance for chronic sublethal sediment bioassays.

Sensitivity Analysis

Demographic modeling techniques can even be used to design more accurate and cost-efficient chronic sublethal bioassays. Sensitivity analysis applied to matrix population models can help identify which life stages or endpoints (for example, survivorship, growth, or reproduction) are most important to population growth in the test species (Caswell 1989). If sensitivity analysis indicated that changes in survivorship of early life stages or perhaps early reproduction had the largest effect on population growth, then tests could be designed to concentrate on those endpoints. Such an approach could reduce the effort and costs incurred in performing a test while also increasing test accuracy.

Conclusions

Current laws and regulations governing the disposal of dredged material emphasize the importance of maintaining the ecological health of the environment. Current guidance for testing dredged material using sediment bioassays makes use of acute lethality tests for identifying potential threats to environmental health. Heightened awareness of the potential long-term effects of chronic low-level exposures to contaminated sediments has generated interest in the development of chronic sublethal bioassays. The major source of uncertainty in the design, performance and results of sediment bioassays concerns predicting the behavior of complex systems (for example, populations) using simple systems (for example, individual animals in a beaker). Use of demographic modeling will enhance the predictive capabilities of chronic sublethal sediment bioassays through providing an ecologically meaningful way of interpreting results and designing future bioassays.

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Environmental Effects of Dredging Technical Notes



Initial Comparisons of Six Assays for the Assessment of Sediment Genotoxicity

Purpose

This technical note reports and compares initial results of six genotoxicity bioassays applied to dredged sediments and describes progress toward development of a testing protocol to aid in regulatory decisionmaking when genotoxic chemicals are an issue of concern.

Background

The Long-term Effects of Dredging Operations Program work unit "Genotoxicity of Contaminated Dredged Material" was initiated in fiscal year 1990 to develop methods for assessing the genotoxic potential of dredged sediments. The impetus driving this new research and development effort was specific regulatory language in section 103 of the Ocean Dumping Act (Marine Protection, Research, and Sanctuaries Act (MPRSA) of 1972) prohibiting the open-water discharge of "mutagenic, carcinogenic, or teratogenic" substances in other than trace amounts, and language less specific but of similar intent in section 404 of the Clean Water Act (CWA).

At the time the genotoxicity work unit was begun, few tests of this kind had been applied to dredged sediments, and none were well understood or generally accepted. It was apparent that with a statutory mandate on the books, the unavailability of technically sound methods for addressing genotoxic potential in sediments constituted a regulatory time bomb.

At a workshop held at the U.S. Army Engineer Waterways Experiment Station (WES) (Reilly and others 1990), participants evaluated the state of the art in genetic and developmental aquatic toxicology and agreed upon an approach that would lead to interpretable and meaningful genotoxicity testing methods

for dredged sediments. Since that time, efforts have been made at WES to adapt and test a suite of methods that show the highest potential for sediment genotoxicity testing in terms of ease of use, interpretability, reliability, and capability for application within the tiered testing framework established in the testing manual for section 103 of the MPRSA, the "Green Book" (USEPA/USACE 1991) and the draft Inland Testing Manual (USEPA/USACE 1994) for section 404 of the CWA.

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Note: The contents of this technical note are not to be used for advertising, publication, or promotional purposes. Citation of trade names does not constitute an official endorsement or approval of the use of such products.

Introduction

Genotoxicity in the strictest sense refers to damage caused by reactions of foreign chemicals with nucleic acids (DNA and RNA) of cells (Jarvis, Reilly, and Lutz 1993). The results may be manifested as mutations, cancers, or developmental abnormalities if the damage is not repaired by the cellular defenses of the organism. Many environmental contaminants are genotoxic, and aquatic organisms such as polychaetes and particularly fishes are highly susceptible to genotoxicities. Aquatic crustaceans and molluscs are much less susceptible, but are not immune to these kinds of effects.

Not all cancers and developmental abnormalities are caused by genetic damage. For the purposes of testing dredged materials, the distinction is important only in test methods development, not in their application. Consequently, the suite of tests being developed at WES for detecting contaminants that are "mutagenic, carcinogenic, and teratogenic" in dredged sediments will include both biochemical endpoints, which are most appropriate for detecting damage to genetic material, and morphological endpoints, which are observable in early life stages of whole organisms. In addition, long-term testing methods where fish are exposed to cancer-causing chemicals and observed for the development of neoplasms and other cancerous lesions are under development at the U.S. Army Biomedical Research and Development Laboratory, Fort Detrick, MD. Although these latter methods may not be suitable for routine testing of dredged sediments, they provide a means for assessing the predictive capability of sediment genotoxicity bioassays intended for use in a regulatory framework.

Genotoxicity tests can be grouped into three categories: general indicators of genotoxic potential, biomarkers of exposure to genotoxic agents, and integrators

of genotoxic effects (Reilly and others 1990). General indicators of genotoxic potential can be applied to aqueous or organic extracts of sediments. These include bacterial tests of mutagenicity, tests for DNA damage in cell cultures exposed to the sediment extracts, tests for the induction of microsomal enzymes, and cytogenetic methods such as tests for micronuclei, anaphase aberrations, and sister chromatid exchange. The tests that are the subject of this technical note are general indicators of genotoxic potential. The second category, biomarkers of exposure, are tests applied to tissues of organisms exposed to genotoxic agents, as well as analyses for bile metabolites of specific compounds, and tests for the induction of metabolizing enzymes. The third category, integrators of genotoxic effects, includes effects on whole organisms, either aberrant morphologies in embryos and larvae or tumors and cancerous lesions in adults. Research on methods for the second and third categories has not yet been undertaken at WES.

Approach

Indicators

Six procedures representing three types of general indicators of genotoxic potential were selected for evaluation (Table 1). The three types of tests are complementary in terms of information obtained regarding genotoxic potential. The two examples of each type produce similar information. Differences among the types of tests are in the responsiveness to classes of genotoxic agents, sensitivity of the test, ease of performance, and potential for application in a routine testing framework.

Type of Indicator	Test
Mutagenicity	(1) Ames Test (2) Mutatox ¹
DNA strand breaks	(1) Alkaline unwinding assay (2) Single cell gel assay
Enzyme induction	(1) H4IIE in vitro assay (2) P450 Reporter Gene System ¹
¹ Proprietary assay.	

Sediments

Sediments with varying degrees of contamination were selected from the WES inventory for testing with the six bioassays described above. The sediments were Soxhlet extracted according to EPA method 3540 (USEPA 1986), cleaned up on silica gel columns (Warner 1976), and the resulting extracts were solvent-exchanged into DMSO for bioassay.

Comparison of Methods

Mutagenicity

A mutation is a change in the DNA of a cell capable of being passed on to the next cell generation (Klaassen and Eaton 1991). Not all mutations are detrimental to the cell or organism, and a few may be advantageous. Most, however, are either silent or dysfunctional, and some mutations are lethal, causing a critical cellular process to be compromised and the cell or organism dies. A mutation can also lead to altered gene expression, resulting in a variety of possible outcomes, including cancer, immune suppression, teratogenesis, or genetic disorders.

Ames Test. The Ames Test is the most widely used test for mutagenicity. It has been estimated that 80 to 90 percent of the chemicals showing mutagenicity in the Ames Test are carcinogenic in mammals (Maron and Ames 1983). This assay uses selected strains of the bacteria *Salmonella typhimurium*, mutated so that they can no longer synthesize histidine, a vital amino acid. The bacteria and the test material are incubated together and placed in agar that does not contain histidine. Bacteria that live under these conditions have undergone a mutation back to the "wild type" capable of manufacturing their own histidine. The formation of bacterial colonies on the agar indicates that the test material has mutagenic potential. Drawbacks to the test are the requirements for sterile technique, numerous quality control tests, and a relatively high degree of technical expertise in the performance of the test. A chief advantage is a well-developed methodology that includes numerous variations on the test capable of detecting many genotoxic modes of action. The Ames Test is in the public domain, making it widely available to potential users. Strains of the bacteria used in the test are available from Dr. Bruce Ames, University of California, Berkeley.

Mutatox. A proprietary mutagenicity assay was selected for comparison with the Ames Test. Mutatox (Johnson 1992) also uses bacteria, the luminescent *Vibrio fischeri*, which has been mutated so that it is no longer luminescent. Reversion of the bacteria after exposure to a mutagen restores luminescence, which is measured using a luminometer. The standard method used to perform the Mutatox assay is described in detail by the manufacturer (Microbics Corporation 1993). Advantages of the method include its simplicity in the quantitation of the response as the amount of light produced, and the fact that it is a relatively simple and rapid technique. Disadvantages include a much higher start-up cost than is required for the Ames Test and a limited database for the interpretation of results.

Comparison of Results. Mutagenicity test results are shown in Table 2. The Ames assay was performed using 10 sediments from different locations. The Soxhlet extracts were split into two aliquots, one of which was cleaned using silica gel (clean extract) and the other was not cleaned (crude extract), resulting in 20 extracts. Silica gel cleanup removes biogenic polar compounds

Sediment	Ames Assay ¹		Mutatox
	TA100	TA98	
Sandy Hook, Clean	-	-	-
Sandy Hook, Crude	-	-	-
Gowanus Creek, Clean	+	+	+
Gowanus Creek, Crude	-	+	+
Arthur Kill, Clean	+	+	+
Arthur Kill, Crude	-	+	+
Red Hook, Clean	+	+	+
Red Hook, Crude	-	+	Mixed ²
Chicago CDF, ³ Clean	+	+	+
Chicago CDF, Crude	+	+	+
Hamlet City, Clean	-	+	+
Hamlet City, Crude	-	-	-
Oakland Reference, Clean	+	+	+
Oakland Reference, Crude	+	-	+
Oakland Inner, Clean	+	+	-
Oakland Inner, Crude	-	+	+
Oakland Outer, Clean	+	-	Mixed
Oakland Outer, Crude	-	+	+
Oakland Hot, Clean	-	+	-
Oakland Hot, Crude	-	+	+

¹ Positive (+) or negative (-) for mutagenicity.
² Positive and negative results obtained from multiple tests.
³ Confined disposal facility.

that are residuals of the extraction process. The same 20 sediment extracts were sent to Microbics Corporation for Mutatox testing. Table 2 shows that comparable results, both positive and negative, were obtained with most of the 20 sediment extracts using two Ames Test bacteria tester strains (TA98 and TA100) with metabolic activation and the Mutatox system. The TA98 test strain responds specifically to frameshift mutations while TA100 responds to base pair substitutions (Zeiger 1985). Mutatox can detect both types of mutations in addition to compounds that intercalate with DNA (Ulitzer, Weiser, and Yannai 1980, 1981).

Based on sediment analytical data not reported in this technical note, both assays detected mutagenicity in suspect sediments and did not indicate mutagenicity in nonsuspect sediments. For example, Gowanus Creek and Arthur Kill, both of which have relatively high polycyclic aromatic hydrocarbon (PAH)

content in the sediments, were identified as mutagenic by both assays. Conversely, Sandy Hook sediments, which have low concentrations of anthropogenic chemical contaminants, were identified as nonmutagenic by both assays. In two cases (Oakland Inner, Clean and Crude), Mutatox failed to detect mutagenicity identified by both tester strains in the Ames Test. In one other case (Oakland, Hot Clean), Mutatox failed to detect mutagenicity detected by one of the Ames Test strains (TA98) that responds specifically to frameshift mutation.

DNA Strand Breaks

Some genotoxic chemicals act by breaking one or both strands of the DNA molecule (Daniel, Haas, and Pyle 1985). Assays that detect DNA strand breaks generally measure the characteristic unwinding of DNA from the double-stranded form to the single-stranded form that occurs when it is in an alkaline environment. The rate of DNA unwinding is directly proportional to the number of strand breaks in the DNA.

Alkaline Unwinding Assay. In the alkaline unwinding assay described by Daniel, Haas, and Pyle (1985), cells (H4IIE rat hepatoma cells for the work described herein) are incubated with a test compound for 6 hr in culture dishes and then subjected to the assay. Alkaline unwinding is measured fluorometrically using Hoechst 33258 dye, which binds specifically to double-stranded DNA and is expressed as an F value, the fraction of double-stranded DNA remaining after 30 min of unwinding. An advantage of the alkaline unwinding assay is that it is technically simpler to perform than is the single cell gel assay. However, the single cell gel assay appears to have greater sensitivity. Both assays detect the same type of damage.

Single Cell Gel Assay. The single cell gel assay developed by Singh and others (1988) utilizes the alkaline unwinding principle, but H4IIE cells are unwound for 20 min after being embedded in an electrophoresis gel. Double- and single-stranded DNA are separated by electrophoresis and visualized using a fluorescence microscope after staining with ethidium bromide. Start-up cost due to the equipment required is approximately 15-fold greater than required for the alkaline unwinding assay.

Comparison of Results. Testing of the cleaned extracts using the Alkaline Unwinding Assay is ongoing. However, preliminary results for three sediment extracts are shown in Table 3.

Since a lower value indicates DNA damage, the results appear to suggest that Hamlet City and Sandy Hook sediment extracts are genotoxic. However, these data are only preliminary, since too few tests have been performed for adequate evaluation.

Single cell gel assays using cleaned extracts are also ongoing, with preliminary data shown in Table 4 for two sediment extracts analyzed to date: Chicago confined disposal facility (CDF), a contaminated sediment, and Hamlet City, a suspect sediment. These preliminary data indicate that the Chicago

Table 3. Preliminary Results of the Alkaline Unwinding Assay for DNA Strand Breaks Using a Cultured Cell Line Exposed to Three Sediment Extracts

Sediment	Sediment Extract Dilution, percent		
	10 ¹	50	100
Chicago CDF	105.6 ²	112.7	94.4
Hamlet City	96.2	76.9	71.1
Sandy Hook	92.3	81.5	87.7

¹ Extracts solvent-exchanged into DMSO.
² Values indicate fraction of undamaged DNA, expressed as percent of control.

Table 4. Preliminary Results of the Single Cell Gel Assay for DNA Strand Breaks Using a Cultured Cell Line Exposed to Two Sediment Extracts

Sediment	Sediment Extract Dilution, percent		
	10 ¹	50	100
Chicago CDF	400 ²	262	154
Hamlet City	167	300	256

¹ Extracts solvent-exchanged into DMSO.
² Values indicate number of cells damaged per 100 cells, expressed as percent of control.

CDF extract damaged DNA at all concentrations, as evidenced by more strand breaks in all treatments than control, and also produced cytotoxicity at the higher two concentrations, indicated by the decreasing number of DNA strand breaks with increasing extract exposure concentration. The Hamlet City extract was also apparently genotoxic, demonstrating a dose-responsive increase in DNA strand breaks above control levels. Both the single cell gel and the alkaline unwinding assays are undergoing further optimization and evaluation.

Enzyme Induction

Induction (stimulated synthesis) of detoxifying enzymes occurs in metabolically active cells of mammals, birds, and fish exposed to certain classes of chemicals. Cytochrome P-4501A1 (CYP1A1)-dependent monooxygenases are a class of enzymes that are induced by exposure to specific organic chemicals including the PAHs, coplanar polychlorinated biphenyls, dioxins, and furans. These are among the most commonly encountered dredged sediment contaminants, and include carcinogens, procarcinogens, and promoters of carcinogenicity. The induction of specific monooxygenases caused by these compounds can be measured quantitatively and used as biomarkers of genotoxicant exposure in eggs or in whole organisms (Tillet, Giesy, and Ankley 1991) or as general indicators of genotoxic potential in cultured cell lines exposed to sediment extracts.

H4IIE in vitro Assay. The H4IIE in vitro assay uses a rat hepatoma cell line incubated with the test compound and allows time for enzyme induction to occur. Ethoxyresorufin-O-deethylase (EROD) activity is then measured fluorometrically as

a sensitive indicator of enzyme induction. As used in the preliminary experiments reported in this technical note, the test has a lower limit of detection of approximately 10^{-11} g (10 picogram) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD). The test can be standardized against 2,3,7,8-TCDD and the results expressed as toxic equivalents (McFarland, Clarke, and Ferguson 1993). The cell line is public domain and readily available.

P450 Reporter Gene System. The P450 Reporter Gene System (P450 RGS) is based on a genetically engineered cell line, and the assay is proprietary. A human hepatoma cell line (HepG2) is used as the basis of the assay. The HepG2 was modified by insertion of the gene for the firefly luciferase enzyme downstream from the CYP1A1 gene. Activation of the CYP1A1 gene results in the expression of luciferase, which is easily measured using a luminometer (Anderson and others 1993). Advantages of the P450 RGS include a shorter time requirement for the response and greater simplicity in instrumentation needs. H4IIE and P450 RGS measure responses to the same chemicals, but sensitivities appear to differ somewhat. P450 RGS appears to be less sensitive to PAH compounds than is H4IIE, and more sensitive to dioxin-like compounds.

Comparison of Results. Table 5 contains preliminary enzyme induction results for six sediment extracts tested using the H4IIE in vitro rat hepatoma bioassay and P450 RGS. An additional sample preparation step that selectively removes PAHs from extracts, sulfuric acid silica gel (SASG) reactive cleanup, was employed for these tests in addition to testing the silica gel-cleaned extracts. SASG reactive cleanup allows differentiation of the presence of PAHs from other contaminants. The results are expressed as average fold induction, which is calculated as the enzyme activity recorded at the end of the induction period divided by the enzyme activity in cells exposed only to the solvent blank. The P450 RGS average fold induction was measured after 16 hr of exposure and the H4IIE after 24 hr. The differences in the results show that 24 hr is clearly insufficient

Sediment	P450 RGS		H4IIE in vitro Assay	
	Average Fold Induction		Average Fold Induction	
	SASG-Treated	Cleaned Extract	SASG-Treated	Cleaned Extract
Sandy Hook	4	6	1.14	0
Oakland Hot	51	61	1.73	1.92
Hamlet City	41	81	1.45	1.63
Passaic River	85	84	2.76	1.84
Newark Bay	102	107	2.50	3.01
Chicago CDF	110	113	2.88	3.10
TCDD standard	NA ¹	142 ²	NA	1.86 ³

¹ Not applicable.
² 6-picogram exposure.
³ 100-picogram exposure.

for the H4IIE. Typically, the H4IIE cells are given 72 hr for induction to occur before measurements are made, and this time difference points to a clear advantage of the P450 RGS.

The sediment extracts were ranked in the same order of genotoxicity by both bioassays, with Chicago CDF being the most genotoxic and Sandy Hook being the least. The SASG cleanup step altered enzyme induction with both assays, generally decreasing genotoxicity, as would be expected by the removal of PAHs.

From these results it appears that the P450 RGS system is more sensitive than the H4IIE bioassay. However, the H4IIE bioassay was performed using large culture dishes while P450 RGS used a microtitre method. Dr. Donald Tillet reported the H4IIE bioassay to be more sensitive when using a microtitre method (personal communication).

Conclusions and Future Directions

Each of the six bioassays demonstrated genotoxic responses with suspect sediment extracts. Results from the Mutatox and the Ames Test correlated very well, as did results from the P450 RGS and the H4IIE bioassay. The Ames Test is a more established mutagenicity test than is Mutatox, although it is much more technically difficult to perform than Mutatox. However, the start-up cost of Mutatox is much greater than the Ames Test (\$23,000 versus ~\$3000), although the cost per assay is far greater for Ames than for Mutatox (\$1,000-\$3,000 versus \$100). A suggested approach for using these assays would be to use Mutatox for primary screening of sediments with the Ames Test as a confirmation assay.

Data from the single cell gel and the alkaline unwinding assays are sparse and inconclusive, and much additional testing is required for satisfactory comparisons to be made. However, these assays are relatively simple and inexpensive to perform (\$50 per sample) and hold promise as rapid initial screens of sediment genotoxicity.

The enzyme assays are highly sensitive and more specific than the mutagenicity and DNA strand break tests, and provide both confirmatory and complementary information. The P450 RGS is a proprietary assay owned by EMCON Marine Sciences/Columbia Aquatic Sciences and is performed for ~\$200 per extracted sample. The H4IIE bioassay is under public domain and can be performed for about the same price. The technical requirements of the two enzyme assays are the same, both involving the use of sterile technique and cell culture. However, the rapidity of the RGS, possibly greater sensitivity, and simplicity of measurement conferred by the generation of luminescence rather than fluorescence are all advantages over the H4IIE assay.

The results obtained thus far indicate both similarities and differences between pairs of tests requiring further delineation. Before final recommendations can be made, the testing suite must be validated against fish cancer and early life

stage developmental aberration models. The next stage of testing will involve determining the sensitivity and selectivity of the assays, and refining the techniques. The tests must also be performed using aqueous as well as other organic sediment extraction methods to determine whether the potential for genotoxicity detected using Soxhlet extracts can be related to bioavailability.

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Environmental Effects of Dredging Technical Notes



A Chronic Sublethal Sediment Bioassay with the Marine Polychaete *Nereis (Neanthes) arenaceodentata*

Purpose

This note provides a general overview of a new 28-day chronic sublethal sediment bioassay designed for the regulatory evaluation of dredged material. The bioassay uses survival and growth rate endpoints with the polychaete *Nereis (Neanthes) arenaceodentata*. The primary technical reference for this new bioassay is Dillon, Moore, and Reish (in press), upon which this overview is based.

Background

Sediment bioassays are used to assess the aggregate toxicity of sediment-associated anthropogenic chemicals. Historically, these bioassays have measured survival of highly sensitive species following acute exposures (10 days). A new generation of sediment bioassays is being developed in which the subtle, sublethal response of test species is measured following chronic sediment exposures (Dillon 1993).

This sediment bioassay was developed for the regulatory evaluation of dredged material under section 103 of the Marine Protection, Research, and Sanctuaries Act of 1972 (Public Law (PL) 92-532) and section 404(b)(1) of the Federal Water Pollution Control Act of 1972 (PL 92-500), as amended. The bioassay, which utilizes both survivorship and growth endpoints, was designed specifically to assess the toxicity of bedded sediments. Research and test development were targeted for eventual use by the commercial bioassay contracting community. Thus, great emphasis was placed on logistical feasibility, practicality, and low capital start-up and operating costs.

To maximize regulatory utility, interpretive guidance explaining the biological importance of test results was also developed. Although targeted for dredged material toxicity testing, this bioassay can be used in other assessments of sediment quality, including bioaccumulation potential, suspended sediment toxicity, and hazard and risk assessments. A more detailed description of this bioassay is given in Dillon, Moore, and Reish (in press).

Additional Information

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Note: The contents of this technical note are not to be used for advertising, publication, or promotional purposes. Citation of trade names does not constitute an official endorsement or approval of the use of such products.

Test Organism

Natural History

The test organism for this sediment bioassay is the nereid polychaete, *Nereis (Neanthes) arenaceodentata*, hereafter referred to as *Neanthes arenaceodentata*, the name most familiar to toxicologists. *Neanthes arenaceodentata* is widely distributed in shallow marine and estuarine benthic habitats of Europe, North America, and throughout the Pacific (Day 1973, Pettibone 1963, Reish 1957, Taylor 1984, Whitlatch 1977). *Neanthes arenaceodentata* constructs one or more mucoid tubes in the upper 2 to 3 cm of sediment. This deposit-feeder ingests particles up to 70 μm in diameter with a preference for those around 12 μm (Whitlatch 1980).

Life Cycle

The life cycle of *N. arenaceodentata* is well documented (Figure 1) (Reish 1957, Pesch and Hoffman 1983). As worms approach sexual maturity, males and females establish pairs and occupy a common tube. Eggs are deposited by the female within the tube; the male presumably fertilizes the eggs at this time. The spent female soon exits the tube and dies within 1 to 2 days or is eaten by the male. The male remains in the tube to incubate and guard the developing embryos. Development is direct and occurs entirely within the parental tube. Emergent juveniles (EJs) exit the parental tube about 3 weeks after egg deposition. They establish tubes of their own and begin to feed. Juvenile worms grow, and eggs become visible in the coelom of females at about 6 weeks postemergence. The eggs continue to grow in the coelom, and deposition occurs 9 to 13 weeks postemergence to complete the life cycle. The entire life cycle can be completed in the laboratory in 12 to 17 weeks at 20 to 22 °C.

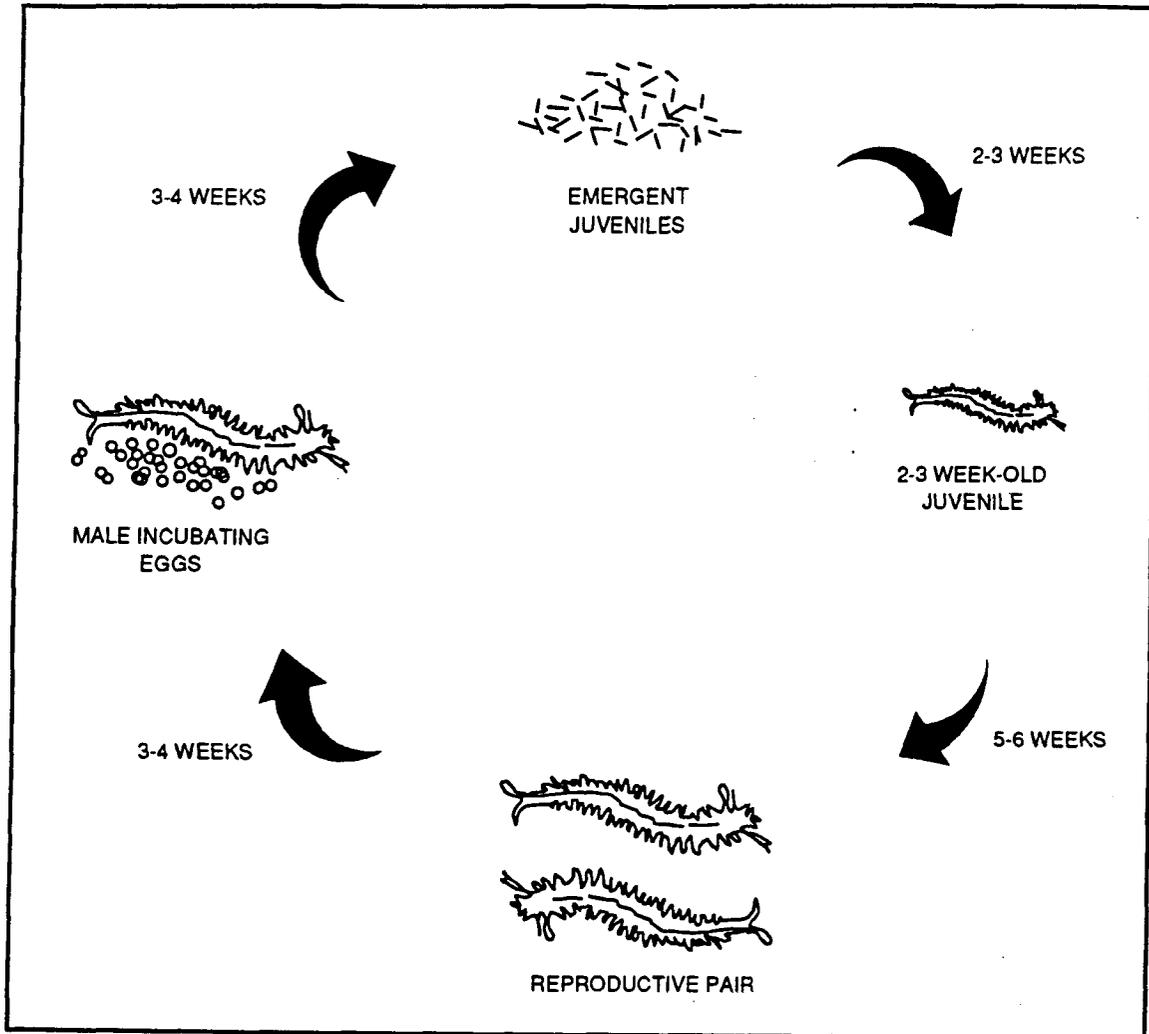


Figure 1. Life cycle of *Neanthes arenaceodentata*

Laboratory Culture Methods

Laboratory cultures of *N. arenaceodentata* were begun at Waterways Experiment Station (WES) in March 1988, from animals provided by Dr. Don Reish of California State University, Long Beach. Worm cultures have been maintained continuously at WES since that time. Worms are maintained at 20 °C in 30 parts per thousand (ppt) artificial seawater made up with reverse osmosis water (ROW). The photoperiod is 12 hr light. EJs are raised to adulthood in 38-L all-glass aquaria (100 EJs/aquarium) containing 30-L aerated seawater and a 2- to 3-cm layer of fine-grain, uncontaminated marine sediment collected near Sequim, WA. Twice weekly, finely ground (≤ 0.50 mm) Tetramarin (100 mg) and alfalfa (50 mg) are added to each aquarium via a seawater slurry.

After 10 weeks, worms are paired using the intrasexual fighting response and the presence/absence of eggs in the coelom (Reish 1974). Pairs are placed in 600-ml beakers with 500 ml of seawater. Each pair is initially fed a slurry containing 4 mg Tetramarin and 4 mg alfalfa. Beakers are monitored daily for

the presence of eggs and EJs. When discovered, EJs are pooled from different broods and returned to the 38-L aquaria to complete the culture cycle.

Toxicology

N. arenaceodentata is recommended for dredged material toxicity testing by the two Federal agencies having regulatory responsibility—the U.S. Army Corps of Engineers (USACE) and the U.S. Environmental Protection Agency (USEPA) (USEPA/USACE 1991, 1993). It has been used in numerous scientific studies designed to evaluate the chronic sublethal effects of contaminated sediment (Chapman and others 1992; Johns, Gutjahr-Gobell, and Schauer 1985; Johns, Pastorok, and Ginn 1991; Pastorok and Becker 1990; Pesch, Mueller, and Pesch 1987; Tay and others 1992). A considerable amount of information has also been reported regarding contaminant-specific toxicity for this species (Moore, Dillon, and Suedel 1991; Reish 1980, 1985).

Statistical Design

The null hypothesis for this sediment bioassay is that there are no statistically significant ($\alpha = 0.05$) differences between the project or test sediments and the reference sediment. Characteristics and selection of an appropriate reference sediment are discussed in USEPA/USACE (1991, 1993). There are five replicates per treatment and five animals per replicate.

Test Protocol

This section describes the protocol for conducting the 28-day chronic sublethal sediment bioassay with *N. arenaceodentata*. General guidance for conducting dredged material toxicity tests can be found in USEPA/USACE (1991, 1993). Supporting information can also be found in Standard Guides produced by the Sediment Toxicology Subcommittee E47.03 of the American Society of Testing and Materials (ASTM 1991a,b).

Sediment Handling and Exposure Vessel Preparation

Sediments are stored cold (4 °C) in sealed containers with a minimum of overlying water. One to 2 days before initiating the bioassay, sediments are removed from cold storage and press-sieved (2-mm screen) without the addition of seawater. After sieving, sediments are thoroughly homogenized. Replicate subsamples are removed from the homogenized sediment for grain size analysis and interstitial salinity, pH, and ammonia determinations. Enough sediment is added to each 1-L beaker to create a 2- to 3-cm layer. After sediment has been added to prelabeled beakers, 30-ppt seawater is slowly added to the 800-ml mark in a manner that minimizes sediment resuspension. Beakers are placed in a temperature- and photoperiod-controlled environment (20 °C and 12 hr light, respectively). Trickle-flow aeration is provided via glass pipette (suspended 2 to 3 cm above the sediment) after any suspended sediment has

settled. Beakers are covered with watch glasses to minimize evaporation and keep out any dust.

Test Initiation

Initial Water Quality. Overlying water should be carefully renewed prior to test initiation. Following this renewal, but prior to the addition of worms, overlying water quality should be determined in each beaker. At a minimum, temperature, salinity, dissolved oxygen, pH, and ammonia should be determined.

Test Organisms. Juvenile worms (2 to 3 weeks old) used to initiate this sediment bioassay are selected from a pool containing about twice the number of animals needed. Selected worms are randomly placed in pre-labeled 100-ml holding beakers (five worms per beaker) containing seawater. The number of holding beakers prepared should be sufficient for the sediment bioassay (five beakers per treatment), initial dry weights (five beakers), and the reference toxicant test (30 beakers).

The bioassay is initiated when worms are introduced into the 1-L exposure beakers containing sediment. Worms are added one at a time to verify the initial census and to visually examine the condition of each worm to ensure the inclusion of representative, undamaged worms.

Feeding. Once the bioassay is initiated, each beaker is provided a seawater slurry containing finely ground (≤ 0.50 -mm) well-hydrated Tetramarin (5 mg) and alfalfa (2.5 mg).

Initial Dry Weights. Initial dry weights are determined on a subsample of 25 worms from the pool of animals used to initiate the bioassay. These worms are placed in five beakers during the selection of test animals (see above). Procedures for dry weight determinations are described below (see **Test Termination** section).

Reference Toxicant Test. A seawater-only 96-hr reference toxicant test with cadmium chloride is conducted at the same time the sediment bioassay is initiated. WES researchers currently use six exposure concentrations (0, 3, 6, 12, 24, and 48 mg Cd/L), five replicate beakers per concentration, and five worms per beaker. Experimental conditions are the same as in sediment bioassays. Worms for the reference toxicant test are drawn from the same pool of animals used to initiate the sediment bioassay. Worms are not fed during the test. After 96 hr, the number of survivors in each beaker is recorded. Water quality is determined in each beaker when the test is initiated and at termination. A 30-ml sample is collected from each beaker initially and at test termination to analytically confirm nominal cadmium concentrations.

Test Maintenance

Each beaker is visually checked every weekday. Abnormal and/or unanticipated events and observations are recorded in the lab notebook. Weekly

seawater renewals are sufficient to maintain good water quality. At each renewal, approximately 80 percent of the overlying seawater is removed and re-filled to the 800-ml mark. Water quality (dissolved oxygen, salinity, pH, ammonia) should be monitored in each beaker prior to each renewal and at test termination. Temperature should be monitored daily. Worms are fed the Tetramarin-alfalfa slurry (described above) twice weekly, after every renewal, and 3 to 4 days later.

Test Termination

The test is terminated after 28 days of sediment exposure. A final visual check of each beaker is made, and terminal water quality is assessed. All surviving worms are removed from each beaker by sieving the sediment through a series of stacked screens (2.0-, 1.0-, and 0.5-mm mesh size). The number of surviving worms per replicate is recorded. All surviving worms from a replicate are briefly rinsed in ROW to remove saltwater and any adhering sediment, pooled, and placed on a tared aluminum weighing pan. Tissue samples are oven-dried at 60 °C to a constant weight (about 24 hr), brought to room temperature under desiccation, and reweighed. Estimated individual worm weights are calculated by dividing the total dry weight biomass in a replicate by the number of survivors. Growth rate (milligrams per day) over the period of the bioassay is calculated by subtracting estimated initial weight from estimated individual final weight and dividing by the exposure period (28 days).

Data Analysis

Data Validation

Data validation procedures generally assume that data are valid until they deviate from some performance criteria. Significant deviations in performance criteria can be grounds for rejecting data unless a good explanation can be provided. Standard data validation procedures for sediment bioassays have not been formalized. Performance criteria for individual sediment bioassays typically evolve over time in an ad hoc fashion rather than by any rigorous numerical analysis. Based on observations at WES and in other laboratories, it is recommended that data validation be carried out using the following assessments.

Performance Criteria for the Negative Control Treatment. The negative control for most sediment bioassays is beakers containing sediment in which the animals were either cultured or field collected. For this bioassay with *N. arenaceodentata*, the recommended performance criteria are ≥ 80 percent survival in any one replicate and ≥ 90 percent mean survival for all replicates containing Sequim Bay sediment. Failure to meet these criteria is grounds for considering the test results invalid.

Performance Criteria for the Positive Control. For this bioassay, the survival of *N. arenaceodentata* in the reference toxicant test with cadmium chloride is

the positive control. Tests conducted thus far indicate that 96-hr LC₅₀s range between 10 and 15 mg/L. These data will eventually be used to construct a Shewart Control Chart, which will be used to identify statistically "out of control" data and, thus, potentially invalid sediment bioassays, when sufficient tests have been conducted. Extant guidance (Environment Canada 1990, Shainin and Shainin 1988) suggests that 15 to 25 tests are required to construct a control chart.

Water Quality Data. Mean water quality should meet the following performance criteria: temperature, 20 °C ± 2 °C; salinity, 30 ppt ± 3 ppt; dissolved oxygen, ≥6.0 mg/L; pH, 8.0 ± 1.0; and total ammonia, ≤1.0 mg/L.

Anomalous Events or Deviations from Good Laboratory Practice. Anomalous events or deviations from good laboratory practice can also be grounds for rejecting data. However, the impact of those events, if sufficient in magnitude or duration, should be reflected in deviations of the above performance criteria.

Statistical Analysis

One-way analysis of variance is used to test the null hypothesis that response in the reference sediment is not statistically different from that in the project sediments. This analysis is conducted for both test endpoints: survival and estimated individual growth rate. Homogeneity of variances is evaluated with either Bartlett's test or Levene's test using appropriate transformations as needed. Normality is evaluated by plotting residuals. Mean separation may be performed via Tukey's HSD test, Dunnett's, or another appropriate parametric procedure. All differences are assumed statistically significant at $P < 0.05$.

Interpreting Bioassay Test Results

A tiered hierarchy for interpreting bioassay test results is recommended, as outlined below.

Tier I: Are the test results valid?

Methods to validate data were discussed above. If the data cannot be validated, and if no reasonable explanation can be provided, test results may be considered invalid. Further analysis would be unwarranted. If the data are acceptable, proceed to Tier II.

Tier II: Are the results statistically significant?

Statistical methods are recommended above. If response in the reference sediment is statistically indistinguishable from that in the project sediments, further data interpretation is unwarranted. If results are significantly different, go to Tier III.

Tier III: Are the results biologically important?

A statistically significant result may or may not be important biologically. For example, if a project sediment causes a statistically significant 5-percent decrease in survival, is that level of response truly detrimental to the organism? Would a 10-percent decrease be twice as "bad" or only incrementally injurious? Deciphering the biological importance of sublethal endpoints such as growth is even more problematic.

For this bioassay, the technical basis for interpreting test results relies on the relationship between growth and reproductive success. Growth and reproduction are energy antagonists. That is, they represent competing demands on a usually limited energy source. As a result, diminished growth will likely lead to adverse effects on reproduction. Establishing the quantitative nature of this relationship provides the technical basis for interpreting the growth endpoint. Moore and Dillon (1993) examined this relationship quantitatively and observed no significant effects on either survival or reproduction when somatic growth rates were ≥ 0.65 mg (wet)/day. Growth rates ≤ 0.45 mg/day resulted in significant reductions in reproduction. Very low growth rates (0.05 mg/day) were associated with a nearly complete cessation of reproduction and very poor survival (5 to 11 percent).

Ultimately, the biological importance of ecotoxicology studies should be interpreted in terms of a meaningful population-level response (Barnthouse and others 1986, Bridges and Dillon 1993, Suter 1990). If a contaminant-induced perturbation represents an important environmental hazard, there is a risk that a local population may decline or even become extinct. This risk can be projected quantitatively using demographic population models. These models represent a tool for integrating life history observations (that is, survival, growth, and reproduction) into deterministic and risk-based estimates of population viability (Bridges and Dillon 1993). WES is currently developing a risk-based demographic model for *N. arenaceodentata*.

Quality Assurance (QA)/Quality Control (QC)

QA/QC represents the administrative and technical steps taken to ensure that reliable data are produced with specified precision and accuracy. Specific QA/QC measures associated with this chronic sublethal sediment bioassay with *N. arenaceodentata* were discussed above. Moore and others (1994) provide general QA/QC guidance for conducting dredged material bioassays.

Test "Ruggedness"

ASTM (1992) defines "ruggedness" as the "insensitivity of a test method to departures from specified test or environmental conditions." For sediment bioassays, "ruggedness" is evaluated from two perspectives: sensitivity to the physicochemical properties of sediments and deviations in normal test conditions

and protocols. Examples of the former include the effects of grain size, interstitial ammonia, presence of indigenous fauna, and organic carbon. These factors are known to bias results of acute lethality sediment bioassays (for example as discussed in DeWitt, Ditsworth, and Swartz 1988), and their potential influence will no doubt increase as test duration increases and more sensitive endpoints are examined. The effects of sediment properties and deviations from normal conditions on survival and growth in *N. arenaceodentata* have been examined (Dillon, Moore, and Gibson 1993) and are summarized below.

Intraspecific Densities

Survival was high (81 to 100 percent) after 6 weeks in 600-ml beakers containing sediment and up to 12 juvenile worms. Growth after 6 weeks was unaffected at densities ≤ 4 worms/beaker but significantly depressed at densities ≥ 8 worms/beaker. In the absence of sediment, the adverse effects of intraspecific interactions were magnified.

Grain Size

Juvenile worms can tolerate a wide range of grain sizes. Survival was high (89 to 100 percent) and unaffected after 6 weeks in grain sizes ranging from 5 to 100 percent sand. Likewise, there was no significant effect on growth. However, there was a consistent trend of reduced worm weight with increasing grain size. This may indicate a possible grain size effect with longer exposures (>6 weeks).

Salinity

Neanthes arenaceodentata is cultured in 30-ppt seawater at WES. Test sediments may come from areas where the salinity is lower. Survival and growth of *N. arenaceodentata* after 6 weeks was unaffected following acute transfers from 30-ppt seawater to salinities ≥ 20 ppt. However, no juvenile worms survived acute transfers to ≤ 15 ppt. The effects of gradual acclimation have not been examined.

Ammonia Toxicity

Juvenile worms exhibited a sharp threshold response to chronic ammonia concentrations, similar to that observed for salinity. Survival and growth were unaffected following a 6-week exposure to total ammonia concentrations ≤ 10 mg/L. Survival was 0 percent at ≥ 40 mg/L. At the intermediate test concentration (20 mg/L), both survival and growth were slightly but not significantly diminished.

Hydrogen Sulfide Toxicity

Survival of juvenile worms in short-term (96-hr) exposures was 100 percent at sulfide concentrations ≤ 5.0 mg/L. At 10.0 and 20.0 mg/L, survival was 44 and 0 percent, respectively.

Resistance to Hypoxia

Survival of juvenile worms in short-term (96-hr) exposures was 100 percent at oxygen concentrations ≥ 1.5 mg/L. At 1.0 and 0.5 mg/L, survival was 68 and 0 percent, respectively.

Future Activities

Although a test protocol can be recommended at this time, additional test development is required. This work falls into the following categories:

- Conduct bioassays on a wide range of dredged material.
- Continue to evaluate test "ruggedness."
- Evaluate interlaboratory variation.
- Compare with other dredged material bioassays.
- Develop a risk-based demographic population model.

Summary of Bioassay Test Protocol

A protocol for conducting a 28-day sediment bioassay with the marine polychaete *Neanthes arenaceodentata* is described. Primary target application is the regulatory evaluation of dredged material. Bioaccumulation potential may also be evaluated under certain conditions. Test endpoints are survival and estimated individual growth rate. The bioassay is conducted at 20 °C under a 12-hr photoperiod in 1-L glass beakers containing aerated seawater (30 ppt) and a 2- to 3-cm layer of bedded sediment. The test is initiated by randomly adding juvenile worms (2 to 3 weeks old) to beakers (five worms per beaker and five beakers per treatment). Worms are quantitatively fed, twice weekly, a seawater slurry containing finely ground Tetramarin and alfalfa. Seawater is renewed weekly (ca. 80 percent volume replacement). Water quality is monitored at least weekly prior to each renewal and at termination. After 28 days, worms are removed via sieving, and the number of survivors in each beaker is recorded. Survivors from each treatment are pooled, placed on a tared weighing pan, dried to a constant weight (24 hr at 60 °C), and weighed to the nearest 0.01 mg. Estimated individual worm weight is determined for each replicate by dividing total dry weight biomass by the number of survivors. Growth is expressed as a rate (milligrams dry weight per day) for each replicate by subtracting initial dry weight from final values and dividing by the exposure period (28 days). Quality control performance criteria for positive and negative controls are reported. Interpretive guidance for this bioassay is based

on the relationship between growth and subsequent reproductive success. Although designed for the regulatory evaluation of dredged material, this bioassay can be applied to other assessments of sediment quality.

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Environmental Effects of Dredging Technical Notes



Fluoranthene K_{DOC} in Sediment Pore Waters

Purpose

This note describes laboratory testing conducted to examine the partitioning of fluoranthene to dissolved organic carbon in the pore water of sediments.

Background

The U.S. Environmental Protection Agency (EPA) is authorized to develop and implement sediment quality criteria (SQC) under Section 304(a) of the Clean Water Act. Under this authority the EPA is proceeding with development of SQC for nonpolar organic compounds and metals. A major assumption in the current approach to SQC is that truly dissolved concentrations of hydrophobic organic contaminants (HOCs) in sediment pore water can be computed by assuming that K_{DOC} (partition coefficient normalized to colloidal plus dissolved organic carbon, DOC) equals K_{oc} (partition coefficient for sediment organic carbon).

The U.S. Army Corps of Engineers is presently investigating the link between contaminant levels in sediment and sediment geochemistry, as well as the utility of equilibrium partitioning approaches for predicting toxicity. In the equilibrium partitioning approach, toxicity is related to pore water concentrations of contaminants. This study compared measured concentrations of truly dissolved fluoranthene to concentrations predicted by current models.

Additional Information

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Introduction

Concentrations of truly dissolved organic contaminants in sediment pore water are regarded as the contaminant fraction available for organism uptake (Landrum and others 1985; Kukkonen, McCarthy, and Oikari 1990). However, concentrations of truly dissolved organic contaminants in pore water are difficult to measure directly because organic contaminants are also associated with dissolved and colloidal organic matter (Carter and Suffet 1982, Chiou and others 1986, Kile and Chiou 1989).

Concentrations of truly dissolved organic contaminants in pore waters are commonly estimated (DiToro and others 1991) by assuming constant partitioning between pore water (truly dissolved) and organic carbon in pore water. However, DeWitt and others (1992) showed that the dissolved organic matter partitioning coefficient (K_{DOC}) was not constant in a base substrate amended with different sources of organic matter. In addition, the structure and composition of humic materials is known to affect K_{DOC} values of hydrophobic organic compounds (Gauthier, Seitz, and Grant 1987; McCarthy, Roberson, and Burrus 1989; Davis 1993). Brannon and others (1991) and Magee, Lion, and Lemley (1991) have shown that changing DOC composition was a primary cause of nonconstant partitioning during facilitated transport of HOCs. Although Brannon and others (1991) did not determine values of K_{DOC} , their data indicated that DOC had lower sorption potential than sediment organic carbon for polychlorinated biphenyls.

If K_{DOC} is not equivalent to K_{oc} , then many of the assumptions used in modeling interactions between HOCs and particulate and dissolved organic carbon in aquatic systems are invalid. This study was conducted to examine the constancy of K_{DOC} in sediment pore water and to compare measured values of K_{DOC} with computed values derived from K_{oc} .

Materials and Methods

Pore waters from 11 sediments were extracted under a nitrogen atmosphere to maintain anaerobic conditions (Brannon and others 1991). The pore water was stored in amber bottles until tested (within 1 day of sample generation). Separate subsamples were acidified with sulfuric acid and set aside for analysis of DOC.

Nine 10-ml aliquots of each pore water sample were removed and placed in a 25-ml glass centrifuge tube. Aliquots from each pore water were spiked with ^{14}C -labeled fluoranthene at three concentrations (0.127, 0.077, and 0.04 mg/L) in each of three replicates. The highest concentration was less than 50 percent of the aqueous solubility of fluoranthene cited in Verschueren (1983). Total fluoranthene in solution was determined by liquid scintillation-counting the original filtered leachate. Additional centrifugation removed the majority of colloidal-bound fluoranthene, while passage through the C-18 Sep-Pak provided a measure of the concentration of truly dissolved fluoranthene.

Truly dissolved means not associated with colloidal material and DOC remaining in the solution after centrifugation.

The total organic carbon (TOC) content of the centrifuged, filtered pore water was determined using a Shimadzu Total Organic Carbon Analyzer, model 5050. The TOC determined for the centrifuged, filtered pore water was designated as the DOC fraction and was used to calculate the K_{DOC} .

Results

The TOC concentration in pore waters ranged from 4.8 to 177 mg C/L (Table 1). Sediments from which the pore waters were obtained represented a range of sites and contents of TOC (Table 1).

Partitioning, or distribution of a hydrophobic organic compound between DOC and water at equilibrium, is mathematically described as follows:

$$K_{\text{DOC}} = C_{\text{DOC}}/C_{\text{W}} \quad (1)$$

Table 1. Sediment Total Organic Carbon (TOC), Pore Water Dissolved Organic Carbon (DOC) Concentrations, and Coefficients for Regression (n = 11) of Truly Dissolved and Bound Fluoranthene in Sediment Pore Water						
Sediment	Sediment TOC, %	Pore Water DOC, mg/L	Slope	Standard Error of Slope	Intercept	r²
Oakland Inner Harbor, CA	0.34	4.8	36,000	6,400	536	0.84
Richmond Harbor, CA	0.49	9.1	55,100	11,700	444	0.76
West Richmond Harbor, CA	0.23	4.2	96,200	11,900	-1,042	0.90
Pinole Shoals, CA	0.52	16.0	35,300	5,300	-304	0.86
Hamlet City Lake, NC	4.76	7.2	106,300	4,200	-54	0.99
Browns Lake, MS	0.84	13.0	86,700	9,700	-277	0.92
Eau Galle Lake, WI	1.42	10.0	61,200	10,300	-142	0.83
Eau Plaine Lake, WI	1.15	177.0	66,900	6,100	55	0.94
Barataria Bay, LA	21.18	79.0	42,035	2,800	-220	0.97
Swan Lake 1, MS	2.37	42.0	44,200	3,900	-275	0.95
Swan Lake 4, MS	2.24	15.0	14,600	2,200	655	0.89

where

K_{DOC} = distribution coefficient, L/kg

C_{DOC} = concentration of contaminant sorbed to the dissolved organic carbon, mg/kg

C_w = truly dissolved aqueous phase fluoranthene concentration, mg/L

Values of K_{DOC} were computed by regression of sorbed (normalized to DOC concentrations) versus truly dissolved fluoranthene for all pore waters. Slopes, standard error of the slope, y-intercepts, and regression coefficients are also presented in Table 1. Error in estimates of the slopes averaged 13.4 percent for all sediment pore waters. Error included differences between replicates and experimental procedure. Regression coefficients were high, ranging from 0.76 to 0.99, as expected for linear isotherms.

Pore water K_{DOC} values for fluoranthene varied from 14,600 to 106,300 L/kg. This wide variation in K_{DOC} (a factor of 7.3) suggests that the *quality* of organic carbon in pore waters differed and affected K_{DOC} . This is further illustrated by the wide range of DOC normalized concentrations of sorbed fluoranthene that exists for a particular solution concentration of truly dissolved fluoranthene (Figure 1).

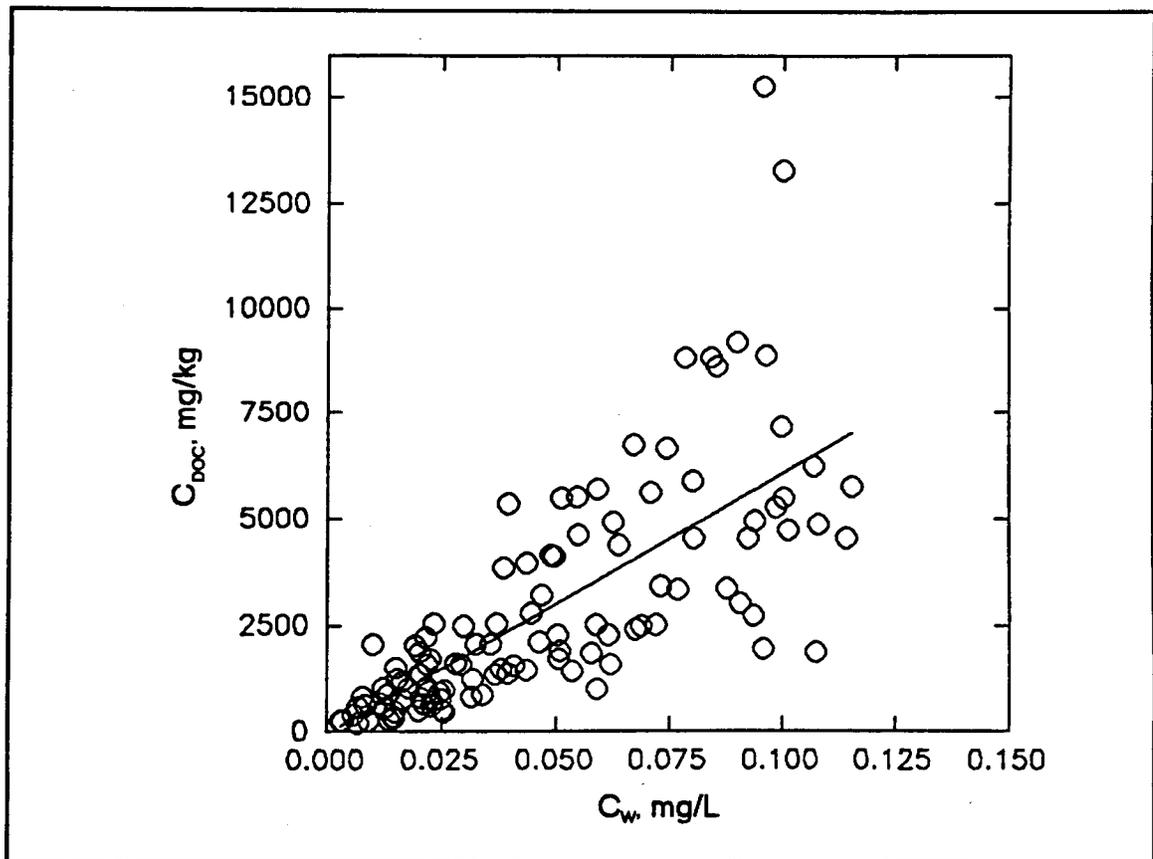


Figure 1. Truly dissolved (C_w) versus DOC normalized (C_{DOC}) fluoranthene concentrations in pore water

As pore water DOC concentrations increased, the fraction of bound fluoranthene increased and the fraction of truly dissolved fluoranthene decreased (Figure 2). The fraction of truly dissolved fluoranthene in solution ranged from approximately 5 to 80 percent of the total fluoranthene in the pore water. This resulted in a wide range of truly dissolved and sorbed fluoranthene concentrations in the experiment.

K_{DOC} was estimated using the common assumption that $K_{DOC} = K_{oc}$ (DiToro and others 1991). The estimated K_{oc} value was computed by substituting K_{ow} or $\log K_{ow}$ (octanol/water partition coefficient) for fluoranthene into the equation $K_{oc} = 0.411 K_{ow}$ (Karickhoff 1981) or $\log_{10} K_{oc} = 0.00028 + 0.983 \log_{10} K_{ow}$ (DiToro and others 1991), respectively. The value of $\log K_{ow} = 5.12$ for fluoranthene was derived by averaging fluoranthene K_{ow} values determined by De Bruijn and others (1989), 5.15, and the U.S. Environmental Protection Agency (1993), 5.09.

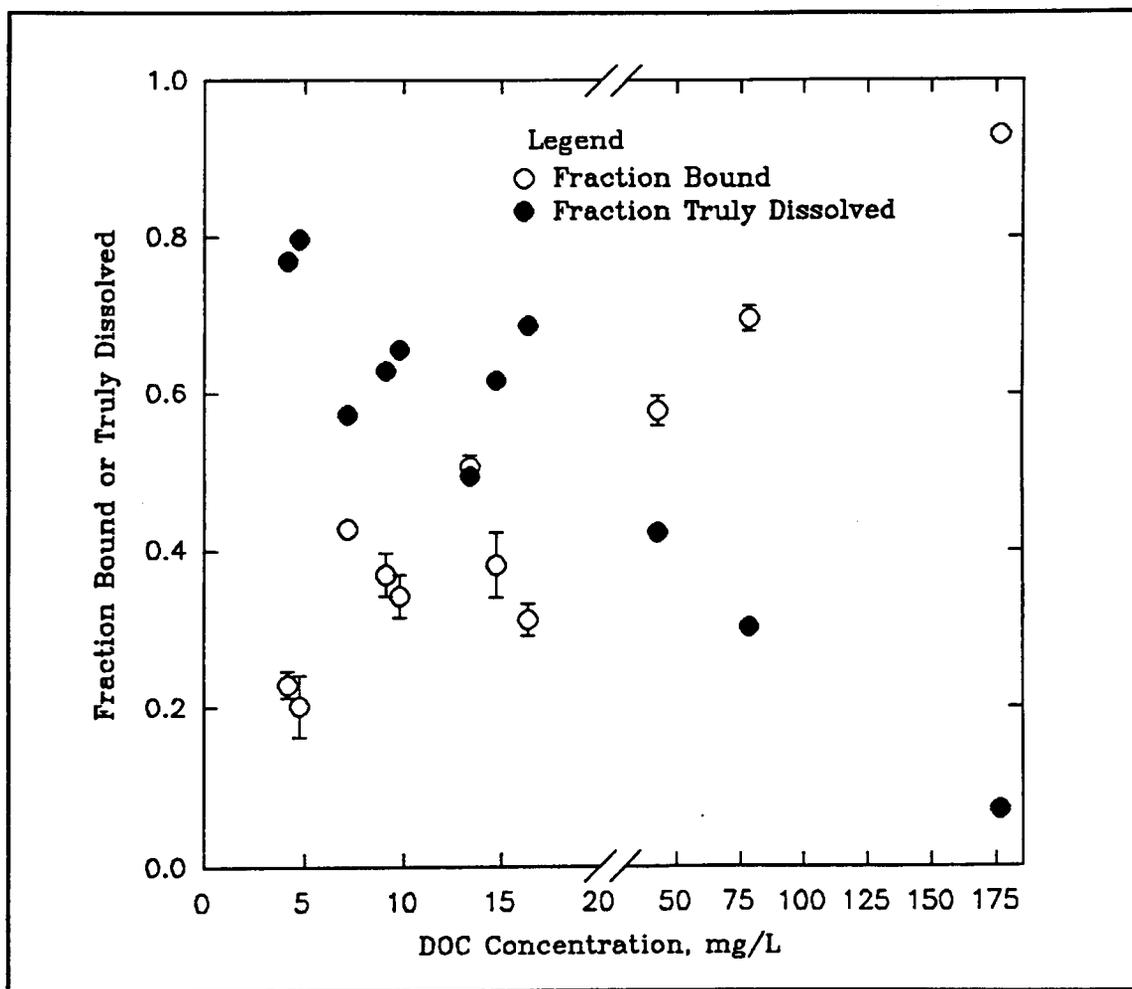


Figure 2. Fraction of bound and truly dissolved fluoranthene as a function of pore water DOC concentration

The measured values of K_{DOC} were consistently lower than the K_{DOC} value estimated using the method of DiToro and others (1991) (Figure 3). The method of Karickhoff (1981) over- and underestimated measured values of K_{DOC} . A particularly wide range of measured K_{DOC} values was observed below 20 mg DOC/L, where most of the pore water DOC values fell.

Concentrations of truly dissolved fluoranthene are related to that bound to dissolved organic matter by the equation (DiToro and others 1991):

$$C_{\text{DOC}} = m_{\text{DOC}} K_{\text{DOC}} C_d \quad (2)$$

where

C_{DOC} = concentration of fluoranthene associated with DOC, mg/L

m_{DOC} = concentration of DOC in solution, kg/L

K_{DOC} = DOC partitioning coefficient, L/kg

C_d = concentration of dissolved fluoranthene, mg/L

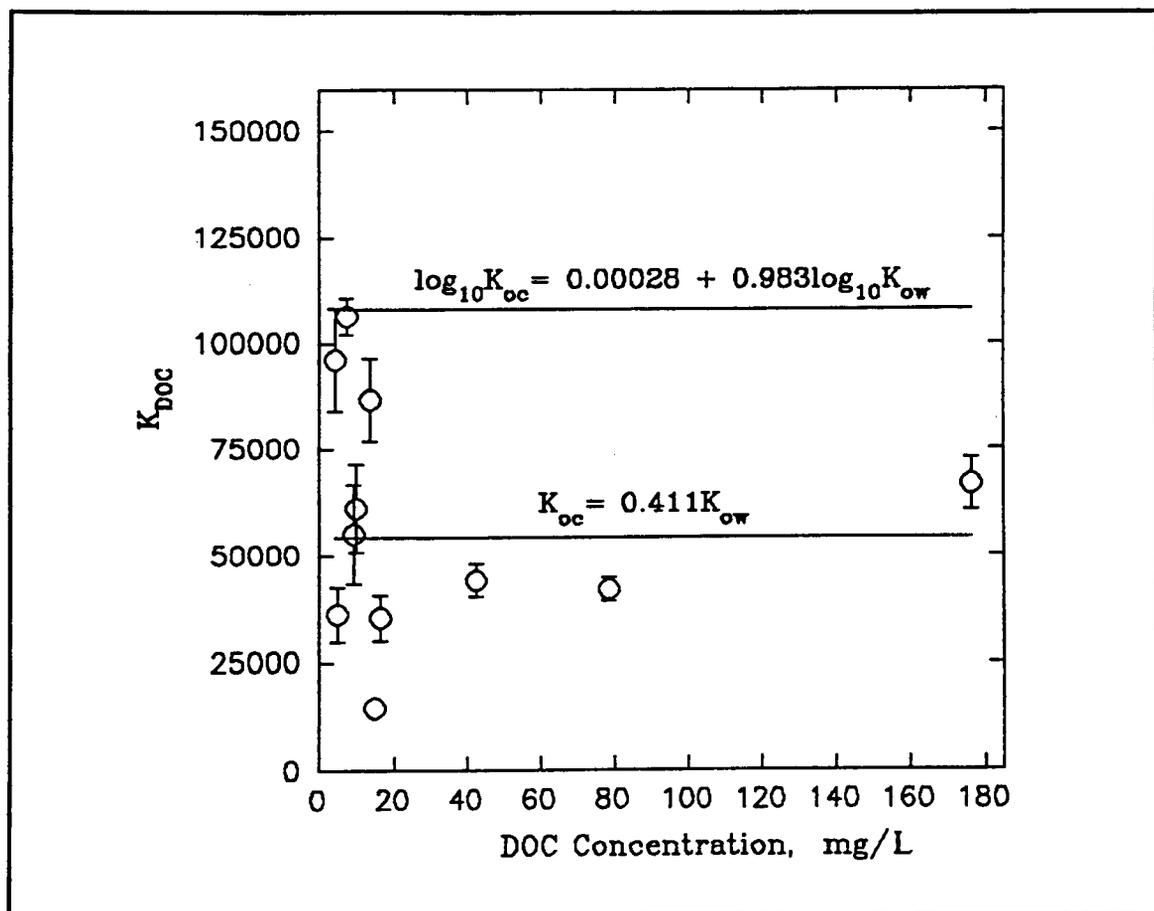


Figure 3. Measured K_{DOC} as a function of pore water DOC concentration. The upper horizontal line represents the DiToro model; the lower horizontal line represents the Karickhoff model. Vertical bars on data points are \pm standard errors of the slope

The concentration of total fluoranthene in solution, C_T , can be expressed as

$$C_T = C_{\text{DOC}} + C_d \quad (3)$$

Substituting for C_{DOC} in Equation 1 yields

$$C_T - C_d = m_{\text{DOC}} K_{\text{DOC}} C_d \quad (4)$$

Solving for C_d gives

$$C_d = C_T / (1 + m_{\text{DOC}} K_{\text{DOC}}) \quad (5)$$

Use of this equation to predict truly dissolved concentrations of HOCs commonly assumes that $K_{\text{DOC}} = K_{\text{oc}}$ (DiToro and others 1991). Measured truly dissolved fluoranthene concentrations were compared to concentrations predicted by Equation 5 (Figure 4) using the methods of Karickhoff (1981) and DiToro and others (1991) for deriving K_{oc} . The value of K_{oc} derived from the equation of DiToro and others (1991) substantially underestimated truly dissolved fluoranthene concentrations. The value of K_{oc} derived from the equation of Karickhoff (1981) more closely approximated measured truly dissolved fluoranthene concentrations, but generally over- or underestimated.

Discussion

Measured values of K_{DOC} in sediment pore waters were not constant and were consistently lower than the value estimated by the method of DiToro and others (1991) and the assumption that $K_{\text{DOC}} = K_{\text{oc}}$. Using the same assumption, the method of Karickhoff (1981) over- and underestimated measured values of K_{DOC} . Differences between measured and estimated K_{DOC} values can be caused by variations in the composition of natural organic matter (Grathwohl 1990, Davis 1993), organic matter aromaticity (Gauthier, Seitz, and Grant 1987), or polarity of pore water organic material (Chiou and others 1986, 1987).

The lack of correspondence of measured values of K_{DOC} with either of the K_{oc} predictive protocols indicates that variations in DOC affect partitioning behavior. Divergence of measured values of K_{DOC} from predicted values indicates that the pore water DOC differs from that of the sediment organic matter for which the relationships relating K_{ow} to K_{oc} were developed. The wide spread of K_{DOC} values measured indicates that the composition of pore water organic matter varied between the sediments investigated. This finding is consistent with several recent reports of wide variation in K_{DOC} values measured in sediment pore water DOC with polycyclic aromatic hydrocarbons (PAHs) (Chin and Gschwend 1992) and soil water soluble DOC with PAHs (Herbert, Bertsch, and Novak 1993).

Evaluation of potential environmental impacts of sediments often involves prediction of truly dissolved concentrations of HOCs in pore water (DiToro

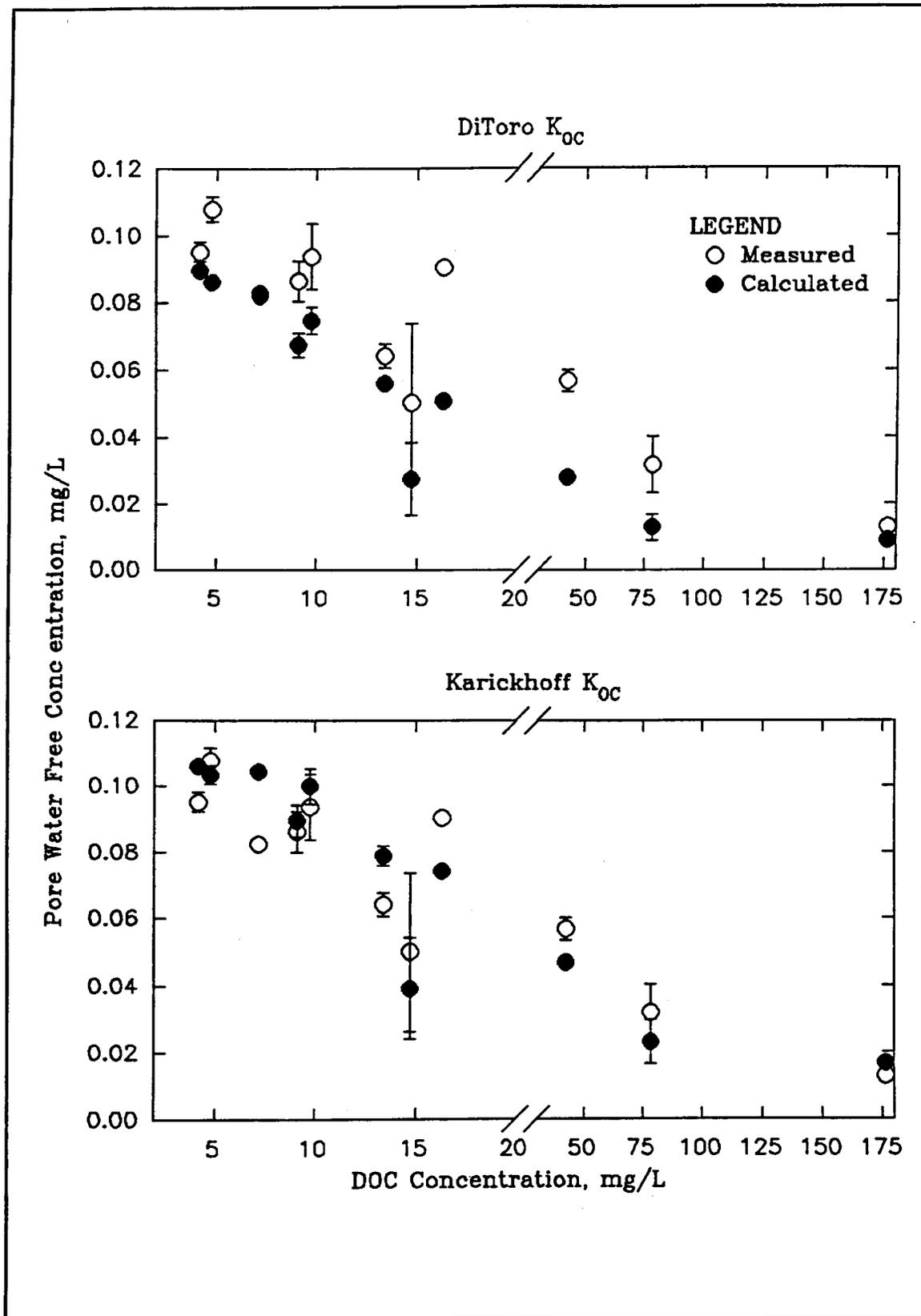


Figure 4. Calculated and measured concentrations of truly dissolved fluoranthene in pore water of varying DOC

and others 1991). Truly dissolved contaminants are usually considered to be the toxic and biologically available fraction of sediment HOC (Landrum and others 1984, Bitton and others 1986).

The divergence of K_{DOC} from values predicted by K_{ow} has implications for prediction of truly dissolved HOCs in pore water. Use of the relationship developed by DiToro and others (1991) relating K_{ow} to K_{oc} consistently underestimated truly dissolved fluoranthene in pore waters. The relationship of Karickhoff (1981) both over- and underestimated fluoranthene pore water concentrations. Use of K_{oc} values to predict truly dissolved concentrations of HOCs in pore water can result in misjudgment of potential environmental impacts.

This study investigated the interactions of only fluoranthene with pore water organic carbon. However, pore water concentrations of other nonpolar organic compounds are likely to exhibit similar behavior (Schrapp and Opperhuizen 1989). Models that rely upon the assumption that $K_{\text{oc}} = K_{\text{DOC}}$ in sediment pore waters should be used with caution until the value of K_{DOC} for sediment pore water can be verified experimentally. The method of Karickhoff (1981) for estimating K_{oc} from K_{ow} in combination with Equation 4 most closely approximated measured K_{DOC} . As illustrated in Figure 2, the spread in sorbed concentrations for a given value of truly dissolved fluoranthene is too extreme to generate an empirical K_{DOC} for pore water. These results are similar to those of Chin and Gschwend (1992), who concluded that an assessment of K_{DOC} must be made on a case-by-case basis when prediction of HOCs in a sediment system is required.

Attempts to extend models developed for the sorption of HOCs by soil and sediment organic matter to dissolved humic substances have been unsuccessful because they consider only the quantity of organic carbon present (Davis 1993). Further study must be directed into the causes of the divergence of measured and estimated values of K_{DOC} .

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Environmental Effects of Dredging Technical Notes



Guidelines for Statistical Treatment of Less Than Detection Limit Data in Dredged Sediment Evaluations

Purpose

This technical note provides recommendations for methods of handling less than detection limit data to permit statistical comparisons of sediment contaminant or bioaccumulation samples in dredged sediment evaluations. Ten censored data methods are evaluated; performance depends upon data characteristics such as equality of variances, type of frequency distribution, and the proportion of the data that is below detection limit.

Background

Regulatory evaluations of dredged sediments frequently require managers to assess contaminant concentrations in the sediments themselves, or in the tissues of organisms exposed to those sediments, as part of a tiered testing protocol (U.S. Environmental Protection Agency/U.S. Army Corps of Engineers (USEPA/USACE) 1991, 1994). A typical Tier III assessment, for example, includes comparison of contaminant bioaccumulation in organisms exposed to the dredged sediment(s) with bioaccumulation in organisms exposed to a reference sediment. Statistical procedures for performing such comparisons are described in detail in Appendix D of the Inland Testing Manual (USEPA/USACE 1994). However, most statistical protocols of the Inland Testing Manual cannot be applied directly in the common situation where some contaminant concentrations are reported only as less than some numerical detection limit (DL). The actual concentrations of these "censored" data are unknown and are presumed to fall between zero and the DL.

Previous studies (El-Shaarawi 1989; El-Shaarawi and Esterby 1992; Gaskin, Dafoe, and Brooksbank 1990; Gilliom and Helsel 1986; Gleit 1985; Haas and Scheff 1990; Helsel 1990; Helsel and Cohn 1988; Helsel and Gilliom 1986; Kushner 1976; Newman and others 1989; Porter and Ward 1991) have examined a variety of methods for handling data that include nondetects. Some of

these studies identified methods that perform well in parameter estimation problems, for example, when a mean contaminant concentration must be estimated to determine compliance with air or water quality standards. Censored data methods recommended for estimation are based on maximum likelihood and regression procedures. However, there is no consensus on which censored data methods should be used when samples must be compared with each other, as in the Tier III bioaccumulation assessments mentioned above, and accurate parameter estimation is unnecessary. The most commonly used methods are the simplest techniques, namely deletion of nondetects or substitution of a constant such as zero, DL, or one-half DL (DL/2) for the unknown observations. Interim guidance in the draft Inland Testing Manual recommended substitution of DL/2 until statistically validated guidelines could be developed.

To address the need for censored data guidelines for sample comparisons in dredged sediment evaluations, a simulation study was conducted to assess the performance of 10 censored data methods. The study procedures and general results have been described elsewhere (Clarke 1994, 1995). The 10 censored data methods are described in this technical note, with recommendations regarding which method to use in specific situations.

Additional Information

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Introduction

Monte Carlo simulations were conducted to evaluate the performance of 10 censored data methods using the statistical procedures recommended in the Inland Testing Manual (USEPA/USACE 1994, Appendix D). Specifically, this entailed comparison of one or more dredged sediments with a reference sediment using the Least Significant Difference (LSD) test on untransformed, log-transformed, or rankit-transformed data (refer to the decision tree, Figure D-5A,B of Inland Testing Manual).

Simulations were conducted using equal and unequal variances with several sample sizes, statistical population distributions, and numbers of sediments to be simultaneously compared with a reference. Censoring was imposed at a "detection limit" equivalent to 20, 40, 60, 80, or 95 percent of the reference sediment population for each set of simulations; uncensored data were also analyzed. Parameter specifications for the simulations are described in detail in Clarke (1995). The entire focus of the study was on small sample size,

necessitated by the high cost of contaminant residue chemical analysis; equal and unequal sample sizes ranging from three to eight replicates were used in the simulations. A total of 335,000 simulations were performed. Simulation results were verified using 271 comparisons of actual chemical concentration data from sediment and tissue samples analyzed for several dredged material contaminant evaluation projects (Clarke 1995).

In the simulations and verifications, censored data methods were evaluated for power and for type I statistical error rate (α). Power is the probability of the statistical test (in this study, the LSD test) to detect true significant differences. Type I error rate is the probability of the statistical test to falsely detect as significant a difference that does not exist in the populations from which the samples were drawn. By convention, α is generally set to 0.05 in biological testing, that is, a false positive error rate of 5 percent or less is considered acceptable. Ideally, power should be about 95 percent, but this is frequently impossible due to fiscal or logistical constraints on the number of samples that can be collected or analyzed. Censored data methods should be chosen to maximize power, and if possible, minimize α . Although all methods can be expected to lose power as the amount of censoring increases, the best methods should minimize loss of power and inflation of α with increased censoring.

Censored Data Methods

Ten censored data methods amenable to simulations using SAS® (SAS Institute, Inc. 1988a,b,c) were chosen for evaluation:

- **DL.** Substitution of the detection limit for all nondetects.
- **DL/2.** Substitution of one-half the detection limit for all nondetects.
- **ZERO.** Substitution of zero for all nondetects.

When data are subsequently transformed to rankits, the above three methods produce the exact same results (assuming all uncensored observations in the sample are greater than DL), and are called **CONST** for substitution of any constant between 0 and DL.

- **UNIF.** Nondetects are replaced by ordered observations x_i ($i = 1, 2, \dots, nc$, where nc is the number of censored observations in the sample) between 0 and DL, where

$$x_i = DL(i - 1)/(nc - 1)$$

and $x_i = DL/2$ when $nc = 1$. This produces a uniform distribution symmetric around $DL/2$ (Gilliom and Helsel 1986).

- **UNIFR.** Replacement of nondetects by random numbers from a uniform distribution between 0 and DL. This may be done using a random numbers table or a random number generator such as the RANUNI function in SAS (SAS Institute, Inc. 1988c).

- **MLE NORM.** Maximum likelihood estimation of below-DL values assuming a normal distribution, using the SAS LIFEREG procedure (SAS Institute, Inc. 1988a).
- **MLE LOGN.** Maximum likelihood estimation of below-DL values assuming a lognormal distribution, using the SAS LIFEREG procedure (SAS Institute, Inc. 1988a).
- **MLE WEIB.** Maximum likelihood estimation of below-DL values assuming a Weibull distribution, using the SAS LIFEREG procedure (SAS Institute, Inc. 1988a).

In the three MLE methods, the $i = 1, 2, \dots, nc$ censored observations are replaced by the values corresponding to the first nc of n evenly spaced percentiles of the MLE-generated distribution.

- **NR.** Substitution of estimated values from a normal distribution using linear regression of above-DL concentrations versus their rankits (Gilliom and Helsel 1986).
- **LR.** Substitution of estimated values from a lognormal distribution using linear regression of logarithms of above DL concentrations versus their rankits (Gilliom and Helsel 1986, Clarke 1992).

The regression equation calculated in these methods is used to extrapolate values for the censored observations. For LR, antilogs of the extrapolated values are used.

SAS program statements for the methods described above are provided in Appendix D of USEPA/USACE (1994) or can be obtained from the author. Several other censored data methods are available but were considered unsuitable for this study (Clarke 1995). In particular, deletion of censored data is not recommended as it results in excessive loss of information and power as the amount of censoring increases. Slymen, de Peyster, and Donohoe (1994) describe and recommend tobit analysis using the SAS LIFEREG procedure for comparing samples with values below DL in environmental studies. The authors present statistical justification for this method, but it could not be compared with the other methods described in this technical note due to the limitations of SAS LIFEREG output for conducting large numbers of simulations.

Considerations in Selecting the Best Censored Data Methods

Simulation results clearly indicate that no single censored data method works best in all situations. Before selecting a method for treatment of nondetects in contaminant evaluations, the investigator should determine, if possible, certain characteristics of the data. Are variances equal or unequal among the samples being compared? If variances are equal, what is the coefficient of variation ($CV = \text{standard deviation} \div \text{mean}$) of the combined samples? If variances are unequal, do they increase as sample means increase, or do they follow no particular pattern in relation to sample means (mixed variances)? When the samples are combined, are the residuals normally distributed,

lognormally distributed (that is, do they pass the test of normality following log transformation), or nonnormally distributed? The type of data distribution and the variance characteristics appear to have the greatest influence upon the censored data methods. For the limited ranges considered in this study, sample size and number of treatments being compared seem to have less effect upon the censored data methods.

To determine type of data distribution and variance characteristics for censored samples, investigators can apply two or more of the censored data methods described above to obtain a range of possible variances and CVs. The revised data (both untransformed and log-transformed) can then be tested for normality and equality of variances using procedures such as those described in Appendix D of USEPA/USACE (1994).

When samples are severely censored, investigators may be able to make an educated guess concerning distribution and variance characteristics based on uncensored data for the same contaminant or on historical data from the same location. Of the 271 comparisons performed using real chemical data in the verification study, half had equal variances among the samples being compared, while 30 percent had mixed variances and 20 percent had variances proportional to the sample means. Sixty percent of the samples passed the Shapiro-Wilk's test of normality (USEPA/USACE 1994), 25 percent passed when data were log-transformed, and 15 percent failed. Nevertheless, in the absence of information to the contrary, it may be reasonable to assume a log-normal distribution for environmental trace chemical data (El-Shaarawi 1989; Gilliom, Hirsch, and Gilroy 1984; Kushner 1976; Newman and others 1989; Ott and Mage 1976; Porter and Ward 1991; Travis and Land 1990). A normal distribution is unlikely for contaminant concentration data when the CV exceeds 1, as such a distribution would include a fair amount of negative concentrations. For example, a normal distribution contains ≈ 17 percent negative values when the CV = 1 and ≈ 31 percent negative values when the CV = 2.

The next consideration should be the relative importance of power versus type I error rate (α) in the statistical comparisons. The censored data methods were compared based on power adjusted for α (that is, mean power minus mean α). The most powerful methods generally had α in the range of 0.05 to 0.10 for amounts of censoring up through 80 percent, but much higher α at 95-percent censoring. If it is crucial to maintain α at approximately 0.05 or less, it may be necessary to select somewhat less powerful methods in certain cases. In a number of situations, there are no suitably powerful methods with $\alpha \leq 0.05$.

When several methods had adjusted mean power within 0.05 of the uncensored data, priority was given to the simplest method(s). In order of increasing complexity, the censored data methods were constant substitution (DL, DL/2, ZERO), substitution from a uniform distribution (UNIF and UNIFR), regression techniques (NR and LR), and maximum likelihood techniques (MLE LOGN, MLE NORM, and MLE WEIB). In most situations, the simplest methods were also the most powerful.

Table 1. Recommended Censored Data Methods for Small Samples to Be Used in Statistical Comparisons

Amount of Censoring, %	Variances	Distribution	Coefficient of Variation	Data Transformation ^a			
				Log	None ^b	Rankit	
≤20	Equal	All	≤ 0.25	DL	DL	CONST, UNIF	
		Normal	0.26 - 1	DL	DL/2, UNIF, DL	CONST, UNIF	
		Lognormal, Nonnormal	0.26 - 1	<i>DL/2, DL</i>		CONST, UNIF	
			>1	<i>DL/2, DL, UNIF</i>		CONST, UNIF	
	Increase as Means Increase	Normal		ZERO^c	LR	—^d	
		Lognormal, Nonnormal		DL		CONST, UNIF	
	Mixed	Normal		—^d	DL	CONST, MLE NORM	
		Lognormal, Nonnormal		<i>DL^c</i>		<i>MLE WEIB^c</i>	
	21 - 40	Equal	All	≤0.25	DL	DL	CONST, UNIF
			Normal	0.26 - 0.5	DL	DL/2, DL	CONST, UNIF
Lognormal, Nonnormal			0.26 - 1	DL/2		CONST, UNIF	
			>1	<i>DL/2, DL</i>		CONST, UNIF	
Increase as Means Increase		Normal		ZERO, MLE NORM	DL	—^d	
		Lognormal, Nonnormal		DL, DL/2		CONST, UNIF	
Mixed		Normal		<i>DL/2^c</i>	ZERO, DL/2^c	CONST, MLE WEIB	
		Lognormal, Nonnormal		DL		CONST	

(Continued)

^a Method(s) in bold indicate most powerful transformation(s). Methods in italics have mean α between 0.06 and 0.10; nonitalicized methods have mean $\alpha < 0.06$.

^b Untransformed data generally should not be used with lognormal or nonnormal distributions.

^c All methods with acceptable power have $\alpha \geq 0.06$.

^d All methods have unacceptably low power and/or high α .

Table 1. (Concluded)

Amount of Censoring %	Variances	Distribution	Coefficient of Variation	Data Transformation ^a		
				Log	None ^b	Rankit
41 - 60	Equal	All	≤0.25	DL/2	DL/2	CONST
		Normal	0.26 - 1	DL/2	DL/2	CONST
		Lognormal, Nonnormal	>0.25	DL/2		CONST
	Increase as Means Increase	Normal		DL	DL	CONST
		Lognormal		DL/2		CONST
		Nonnormal		DL, DL/2		CONST
	Mixed	Normal		— ^d	— ^d	CONST ^c
		Lognormal		DL/2		CONST ^c
		Nonnormal		DL/2		CONST
61 - 80	Equal	All	≤0.25	DL/2 ^c	DL/2	CONST
		Normal	0.26 - 1	DL/2	DL/2, ZERO	CONST
		Lognormal, Nonnormal	0.26 - 0.5	DL/2 ^c		CONST
			0.51 - 1	DL/2		CONST
		Lognormal	>1	DL/2 ^c		CONST ^c
	Nonnormal	>1	— ^d		— ^d	
	Increase as Means Increase	Normal		DL	DL/2, ZERO	CONST
		Lognormal		DL/2		CONST
		Nonnormal		UNIF		CONST
	Mixed	Normal		— ^d	— ^d	— ^d
Lognormal, Nonnormal			DL/2 ^c		CONST ^c	

Recommendations for Censored Data Methods

Censored data methods recommended for various situations of equal and unequal variances, statistical frequency distributions, CVs, data transformations, and amounts of censoring are given in Table 1. When two or three methods are essentially equivalent in power, type I error rate, and simplicity, all are listed in the table in order of decreasing power. Method(s) highlighted in bold indicate the data transformation(s) having the highest adjusted power in a given situation. Methods in italics have mean α between 0.06 and 0.10; nonitalicized methods have mean $\alpha < 0.06$. When the recommended method has mean $\alpha \geq 0.06$, if possible, an alternative (although usually less powerful) method having lower α is given in the table. Situations in which all methods have unacceptably low power and/or high α are also indicated in the table. Methods having adjusted mean power within 0.05 of the most powerful method for a given censoring percentile and variance-distribution-CV combination and at least half the power of the uncensored data for that combination were considered to have acceptable power.

In most situations shown in Table 1, a single powerful method can be applied regardless of which data transformation, if any, might be needed. For example, when censoring is ≤ 20 percent, variances are equal, and CV is ≤ 0.25 , DL should be substituted for all nondetects. The tests of assumptions in Appendix D of USEPA/USACE (1994) would then determine whether untransformed, log-transformed, or rankit-transformed data should be used in the statistical comparisons. Alternatively, UNIF could be used with rankits. These methods have approximately equal power. However, if censoring is between 40 and 60 percent, variances are equal, and CV is ≤ 0.25 , CONST with rankits should be preferred, as the power of this combination exceeds that of any method with untransformed or log-transformed data. In cases when power is exceptionally low, especially when variances are unequal, a different method for each transformation may be required to maximize power.

Following is a discussion of the individual censored data methods and the situations in which they should or should not be used.

DL is generally the preferred method at low to moderate proportions of censoring, especially when the CV is low, or when variances are unequal and data are not normally distributed. In particular, DL performs better than all other methods and much better than the other simple substitution methods at ≤ 40 percent censoring when the CV is extremely low (≤ 0.25). In most cases DL should not be used with data that are highly censored (> 60 percent censoring). DL has low power at ≤ 40 percent censoring with log transformation when data are normally distributed and variances increase with increasing means.

DL/2 generally begins to surpass DL in power as CV and censoring increase. DL/2 tends to have slightly higher α than DL when variances are equal. DL/2 should not be used when the CV is extremely low (≤ 0.25) and less than

40 percent of the data are censored. DL/2 also has low power and/or high α at ≤ 60 percent censoring when data are normally distributed and variances are unequal.

ZERO is recommended for use with untransformed, normally distributed data in a few situations. In general, ZERO should not be used with log-transformed data as this amounts to deletion of the censored data, resulting in low power and high α . One exception, in which ZERO proved to be the most powerful method with log-transformed data, was normal distribution at ≤ 40 percent censoring when variances increase as means increase. However, α in this case exceeds 0.05.

CONST is almost universally appropriate for rankit-transformed data, and is usually the most powerful method with rankits. In several situations CONST with rankits is equally or more powerful than the best-performing method with untransformed or log-transformed data. However, when data are normally distributed, variances increase with means, and censoring is ≤ 40 percent, all methods with rankits have unacceptably low power compared with log-transformed and untransformed data. Type I error rate is high for CONST with rankits when variances are mixed and data are normally distributed; in almost all other cases, α does not exceed 0.06.

UNIF is the most powerful method with log-transformed data at high amounts of censoring when data are nonnormal and variances increase as means increase. When used with rankits, UNIF is essentially equal in power to CONST in most situations. Type I error rate tends to be extremely low for UNIF, especially as censoring increases. Therefore, UNIF can be a suitable alternative to the most powerful method in some situations when low α is desired.

UNIFR is generally slightly less powerful, with slightly higher α , than UNIF. Power is low for most situations at 60 percent censoring or more. UNIFR is not the recommended method in any situation.

MLE NORM is recommended in two situations as an alternative to the most powerful method when low α is desired: with rankits at ≤ 20 percent censoring when variances are mixed and data are normal, and with log-transformed data at 21 to 40 percent censoring when variances increase with means and data are normal. MLE NORM has low power at 60 percent censoring or more, and also in many cases at 40 percent and even 20 percent censoring. MLE NORM should not be used with log-transformed data when the CV is high as this method may substitute negative concentrations for the nondetects.

MLE LOGN is not the most powerful method in any situation. Power is low when censoring exceeds 40 percent, and α tends to be high for log-transformed data in many cases at low amounts of censoring.

MLE WEIB is recommended for rankits as an alternative to CONST at 21 to 40 percent censoring when variances are mixed, data are normally distributed, and low α is required. MLE WEIB should also be used with rankits at ≤ 20 percent censoring when variances are mixed and data are not normally distributed. In most other cases MLE WEIB has less power than MLE LOGN, and is inappropriate for log-transformed data, or for any data when censoring exceeds 40 percent.

LR and NR appear to be inappropriate as censored data methods for statistical comparisons of small samples in most circumstances. Power is generally low even at 20 percent censoring, and declines precipitously as censoring increases. Conversely, α is generally high even at 20 percent censoring and increases dramatically as censoring increases, sometimes approaching 1. LR is recommended only for untransformed data at 20 percent censoring when variances are proportional to means and data are normally distributed.

The simple substitution (DL, DL/2, ZERO, CONST) and uniform distribution (UNIF, UNIFR) methods can be applied regardless of the amount of censoring. The MLE methods cannot be used when all observations in a sample are below DL. The regression methods (LR, NR) require at least three uncensored observations in each sample, and thus are inapplicable for small sample sizes when censoring exceeds about 20 percent.

Verifications

Verification results overwhelmingly support the simulation study conclusions that simple substitution or uniform distribution methods work best in most situations to prepare censored samples for statistical comparisons. In no case did the maximum likelihood or regression techniques have sufficient power in the verifications to be considered useful. Verification results favor the use of DL at 20 to 40 percent censoring when the CV is low (≤ 0.25), and DL/2 otherwise. Although generally less powerful than DL/2, UNIF and UNIFR have low α and perform well at 20 to 40 percent censoring when log transformation is not used. ZERO also performs well, especially at 40 to 60 percent censoring, but should not be used with log transformation. No methods have sufficient power to be useful at 80 percent censoring except DL/2 when the CV is high (> 0.75).

Summary

Simulation and verification results indicate that, in most cases, the sophisticated statistical techniques recommended for estimation problems involving censored data are unnecessary or even inappropriate for statistical comparisons of small, censored data samples. In general, the simple substitution methods work best to maintain power and control type I error rate in statistical comparisons. Recommended steps in selecting the best censored data method for a given situation are listed below.

For each contaminant for which some data are reported as nondetect or <DL:

- Determine proportion of data that are censored (all samples combined).
- Determine whether variances are equal or unequal among samples. If unequal, do the variances increase as means increase, or are the variances seemingly random (mixed)?
- Determine CV of combined samples.
- Determine whether combined sample residuals are distributed normally, lognormally, or nonnormally. If $CV \geq 1$, assume lognormal or nonnormal distribution.
- Refer to Table 1 to determine most appropriate method given the amount of data censoring, properties of variances, and type of statistical distribution. Where possible, preference should be given to methods in bold.
- If it is crucial to maintain α at approximately 0.05 or less, choose nonitalized methods where available in Table 1.
- Apply selected method to censored data, then continue with tests of assumptions and statistical comparison procedures as outlined in USEPA/USACE (1994). Avoid a data transformation for which no method is given in Table 1 due to low power or excessively high α .
- Do not attempt statistical comparisons of severely censored samples in situations where no censored data methods are considered appropriate. In such cases, the probability of an erroneous outcome is high.

If it is impossible to determine characteristics of the variances or statistical distribution for censored data samples, use DL for up to 40 percent censoring or DL/2 for 40 to 80 percent censoring. An alternative, although somewhat less powerful in many situations, is to substitute any constant between 0 and DL, convert the data to rankits, and then follow the nonparametric decision procedures in Figure D-5B of USEPA/USACE (1994). Power loss using CONST with rankits, when compared with DL or DL/2 on untransformed or log-transformed data, is generally around 5 to 10 percent when variances are equal and data are lognormally or nonnormally distributed, <4 percent when variances are equal and data are normally distributed, up to 14 percent when variances are proportional to the means, and up to 6 percent when variances are mixed. No matter what technique is used, power will generally decline as censoring increases. Beyond 60 to 80 percent censoring, it is unlikely that any technique will perform acceptably.

It is quite possible that an evaluation including a number of sediments and contaminants would produce comparisons involving several different combinations of censoring proportions, variance characteristics, and data frequency distributions. Following the guidelines herein would result in the application of more than one censored data method to the project data. This is entirely acceptable when the censored data methods are selected for the purpose of maximizing power and minimizing type I error. *What is not acceptable is to try several censored data methods for the purpose of finding one that will produce a desired statistical comparison outcome.*

The simulation study did not address the performance of censored data methods in the common situation of multiple detection limits within a set of replicate observations. However, the simple substitution methods shown to work well in nearly all cases with single-detection limit censored samples can be applied without modification to multiple-detection limit censored samples.

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Environmental Effects of Dredging Technical Notes



Methods for the Assessment of the Genotoxic Effects of Environmental Contaminants; Subcellular Effects

Purpose

This technical note is the first in a series of three that outline and describe the principal methods that have been developed to test the potential of environmental contaminants for causing mutagenic, carcinogenic, and teratogenic effects. This technical note describes methods used to discern genotoxic effects at the subcellular level, while the second in the series (EEDP-04-25) describes methods used to discern genotoxic effects at the cellular and organ/organism level. In the third technical note (EEDP-04-26), recent literature citations for each topic are listed to assist readers in locating source information. Technical Note EEDP-04-26 also includes a glossary of terms.

The information presented in these technical notes is intended to provide Corps of Engineers personnel with a working knowledge of the terminology and conceptual basis of genotoxicity testing. To develop an improved understanding of the concepts of genotoxicity, readers are encouraged to review "A Primer in Genotoxicity" (Jarvis, Reilly, and Lutz 1993), presented in Volume D-93-3 of the *Environmental Effects of Dredging* information exchange bulletin.

Background

Many contaminants of dredged material acting singly or in combination are toxic to exposed organisms through effects on DNA. Such effects are usually the result of low-level exposures lasting a long time. These effects can result in reproductive failure of organisms, impaired growth and development (especially of early life stages), and tumors, sometimes cancerous, in fishes and vertebrate wildlife. Collectively, such effects are called "genotoxicity" and result from damage to the genome of a cell. The damage is heritable, that is, passed on to future cell generations upon duplication of the affected cells.

Tests are available to detect genotoxic effects at each level of organism integration: subcellular/molecular, cellular, tissue/organ, and whole organism. These tests, the most important of which are described in these technical notes, have been developed for and applied specifically to the genotoxicity of single-chemical compounds to mammalian species.

Although tests of sediment genotoxicity are not routinely applied in regulatory contexts, the potential for their requirement in special circumstances is implied by the language of public law. For example, the Marine Protection, Research, and Sanctuaries Act of 1972 (PL 92-532), which regulates disposal of dredged material in the oceans, specifically prohibits open-water disposal in other than trace amounts of "known carcinogens, mutagens, or teratogens or materials suspected to be carcinogens, mutagens, or teratogens by responsible scientific opinion." In addition, the emphasis in environmental toxicology over the last decade has increasingly shifted away from the catastrophic endpoint (death of individual organisms in acute exposures) to chronic and sublethal toxicities having the long-range ability to seriously affect the survival and well-being of populations of organisms.

For this reason, research aimed at developing a methodology for testing the genotoxic potential of contaminated sediments to aquatic organisms is being undertaken at the U.S. Army Engineer Waterways Experiment Station. This technical note, prepared under the sponsorship of the Long-term Effects of Dredging Operations (LEDO) Program, provides background information to assist potential users of genotoxicity testing methods in evaluating and interpreting test results.

Additional Information

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Note: The contents of this technical note are not to be used for advertising, publication, or promotional purposes. Citation of trade names does not constitute an official endorsement or approval of the use of such products.

Effects on Genetic Material (Refs. 1-10)*

A mutation occurs when a nucleotide is chemically modified, deleted, or substituted. Certain environmental contaminants act as mutagens in that they

* Refer to bibliographic citations 1 through 10 in *Environmental Effects of Dredging* Technical Note EEDP-04-26.

covalently bind to DNA nucleotides, chemically modifying the DNA. The cell contains DNA repair enzymes that can repair mutations under normal circumstances. However, when the organism is exposed to an excessively high level of a mutagen, the DNA repair enzymes may not be able to repair all of the mutations or may misrepair some mutations by deleting the nucleotide rather than replacing it, or by substituting a wrong nucleotide for the mutated one. Depending on the location of the mutation, the number of mutations, and whether the mutation is repaired by the cellular DNA repair enzymes, a mutation may progress to tumor formation or cancer in the organism.

Two basic types of assays for mutagenicity are available. Bacterial assays such as the Ames assay and the proprietary assay, Mutatox, are designed to detect the presence of mutagenic compounds in a sample. The other type of assay, exemplified by ^{32}P -postlabeling and the alkaline unwinding assay, is designed to determine whether a particular organism or cell has experienced mutations.

Ames Assay (Refs. 11-33)

The Ames assay uses strains of a bacterium, *Salmonella typhimurium*, that have been purposely mutated so that they cannot produce the amino acid histidine, which is required for survival. In order for colonies of these bacteria to grow, they must be cultured on media containing sufficient levels of histidine. For the assay, the bacteria are incubated with the test compound on culture media containing trace levels of histidine and are checked for colony formation. If the test compound is mutagenic, the genetically altered bacteria will reverse mutate, or revert, to the "wild type" and be able to synthesize their own histidine. Thus, bacterial colonies growing on the histidine-deficient media indicate the presence of mutagens in the growth medium. Chemicals that are mutagenic in the Ames assay are usually carcinogenic in life-cycle rodent bioassays.

Mutatox (Refs. 34-44)

Mutatox is a relatively new, easy to perform proprietary assay that uses a dark mutant of the bacterial strain *Photobacterium phosphoreum*, which normally bioluminesces (like fireflies). These dark mutants of *P. phosphoreum*, similarly to the *S. typhimurium* tester strains used in the Ames assay, revert to the wild type in the presence of mutagens. The mutation causes *P. phosphoreum* to bioluminesce, and the light produced is easily measured with a luminometer.

A characteristic of prokaryotes such as *S. typhimurium* and *P. phosphoreum* is that, unlike almost all vertebrate and invertebrate species, they do not contain cytochrome P450. Cytochrome P450 is a family of membrane-bound enzymes found primarily in liver cells that function in steroid metabolism and in the metabolism of foreign compounds. Many environmental contaminants, such as polycyclic aromatic hydrocarbons (PAHs), are not genotoxic in their original chemical form, but can be biotransformed (metabolically activated) to a reactive chemical form by cytochrome P450 enzymes. The Ames assay and

Mutatox can be used to distinguish between contaminants that require bioactivation and those that do not, by inclusion of a rat liver preparation (S9) containing cytochrome P450 with the bacteria and test extract. The Ames assay is typically performed both with and without exogenous metabolic enzymes to differentiate direct-acting mutagens from promutagens, or those that must be metabolized for activity.

³²P-Postlabeling (Refs. 45-50)

³²P-Postlabeling is a highly sensitive method used to determine whether DNA from a particular organism has been chemically modified by a mutagen, that is, has formed adducts. DNA is extracted from the organism, usually either from the liver or the whole organism, and enzymatically digested to a mixture of normal and adducted nucleotide monomers. The nucleotides are then enzymatically labeled with ³²P, and the adducts are enriched relative to the normal nucleotides either enzymatically or by extracting into n-butanol. Adducted nucleotides are quantitated using autoradiography, which entails laying a piece of photographic film over the TLC plate used to separate the different nucleotides and letting the gamma rays from the ³²P label expose the film.

Alkaline Unwinding Assay (Refs. 51-61)

Alkaline unwinding assays are used to indirectly measure adduct formation by determining the number of strand breaks that occur. The DNA from exposed cells or organisms is isolated and subjected to alkaline conditions (pH > 10.5) by the addition of a strong base. The alkalinity simultaneously causes the DNA to break at the sites of most DNA adducts and causes the DNA to unwind from its normal double-stranded configuration to the two single DNA strands. Higher numbers of DNA adducts cause higher numbers of DNA strand breaks which, in turn, causes a faster rate of double-stranded DNA unwinding as compared to normal DNA. The rate of DNA unwinding is calculated and can be expressed as number of adducts per milligram DNA.

A variation of the alkaline unwinding assay is the single cell gel (SCG) assay, also known as the "comet" assay. For the SCG assay, cells (for example, blood cells) from an exposed organism or cells grown in culture and exposed to extracts are embedded into a gel-coated microscope slide. The slide is incubated in an alkaline solution, to allow DNA unwinding, and is subjected to electrophoresis. The cells and DNA are stained and scored with a microscope. Cells with damaged DNA have the appearance of a comet, with DNA trailing from the cell body, while cells with undamaged DNA appear normal.

Unscheduled DNA Synthesis (Refs. 62-65)

Unscheduled DNA synthesis is a test for mutagenicity that monitors DNA repair following DNA damage from a mutagen. Cells (typically, isolated liver cells from an exposed organism or cells exposed in cell culture) are incubated

with ^3H -thymidine, a radiolabeled nucleoside. In repairing the damaged DNA, ^3H -thymidine is incorporated into the DNA. The amount of repair, monitored autoradiographically as in ^{32}P -postlabeling, is proportional to the amount of damage.

Nongenotoxic Effects on Adjunct Systems (Refs. 1-10)

Exposure to genotoxic agents usually affects living organisms in numerous ways other than just by causing DNA damage. The inclusion of observations on adjunct systems concurrently with genotoxicity measurements can contribute substantially to a correct interpretation of the result. For example, a chemical may produce a genotoxic effect such as significant mutation in the Ames assay, while at the same time causing either an increase or a decrease in one or more nongenotoxic parameters. It would be highly desirable to know the effects of increasing or decreasing these parameters with regard to the initiation or promotion of cancer. By understanding the roles played by adjunct systems, changes that occur in their function through exposure to genotoxicants can be interpreted in terms of potential for the development of cancer or abnormal early development.

Cytochrome P450 (Refs. 66-81)

Cytochrome P450 is a group of enzymes located primarily in metabolically active tissues (such as the liver, spleen, kidney, and lungs) that function in steroid metabolism and also metabolize xenobiotic compounds such as PAHs. Exposure to certain groups of compounds including polyhalogenated dibenzo-*p*-dioxins and furans, polychlorinated biphenyls, and PAHs induces the formation of specific cytochrome P450 enzymes that are normally not present or present in very low quantities. This enzyme induction is believed to have a promotional effect on initiated cells, in that it stimulates cells that have been predisposed, or initiated, to cancer formation to become cancerous. Induction of cytochrome P450 also leads to a higher rate of xenobiotic metabolism, which is generally beneficial to the organism. In the case of several of the PAHs, however, metabolism leads to activation of the parent PAH to a reactive metabolite, which can form adducts with DNA, causing mutations.

Knowledge of the mechanism and effects of cytochrome P450 induction can provide tools for screening sediment contamination. Monitoring cytochrome P450 levels in organisms exposed to sediments either in the laboratory or in the field can give an index (biomarker) of contaminant exposure. Cytochrome P450 induction is also the basis for a bioanalytical assay for dioxins, the EROD induction assay.

Bile Metabolites (Refs. 82-85)

Once a toxicant is absorbed into the bloodstream of an organism, it is transported through the liver where a large portion of the toxicant is absorbed into the liver cells (hepatocytes). Hepatocytes are rich in cytochrome P450 and

other metabolizing enzymes, and a portion of the absorbed toxicant is metabolized. In the case of most environmental contaminants, metabolism leads to the formation of unstable metabolites, which usually immediately react with some cellular component in close proximity to the site of metabolism. The majority of these unstable metabolites react with intracellular water, typically forming innocuous secondary metabolites. A fraction of the unstable metabolites reacts with other cellular components, including DNA (forming an adduct) and bile. Bile is the waste product formed by hepatocytes, which is secreted into the gall bladder and finally into the intestinal tract. Bile can be isolated from an organism and analyzed, usually using high performance liquid chromatography, for specific bile metabolites, giving an index of exposure (biomarker) to that particular compound.

Phase II Enzymes (Refs. 86-90)

The enzymes that catalyze the metabolism of xenobiotic compounds are generally classified into two groups, phase I enzymes and phase II enzymes. Phase I enzymes, which include cytochrome P450 monooxygenases (or mixed function oxidases, MFOs), act to expose or add functional groups on the parent xenobiotic molecule, increasing its water solubility. Phase I metabolites often are excreted in the urine. Once a compound undergoes phase I metabolism, or if the parent molecule contains a phase I functional group, the molecule may undergo phase II metabolism, as illustrated in Figure 1. Phase II metabolism adds, or conjugates, a bulky endogenous molecule to the parent molecule. Endogenous molecules that are conjugated in phase II metabolism include glucuronic acid (a glucose derivative), amino acids, sulfate groups, methyl groups, glutathione, and acetyl groups. Phase II metabolism generally facilitates biliary as well as urinary excretion.

Phase II metabolism acts as a protective mechanism for the cell, in that reactive phase I metabolites have an alternative site to bind rather than to DNA. A compromise in phase II metabolism may lead to genotoxic effects. Phase II enzyme systems are saturable; that is, they are overwhelmed when presented

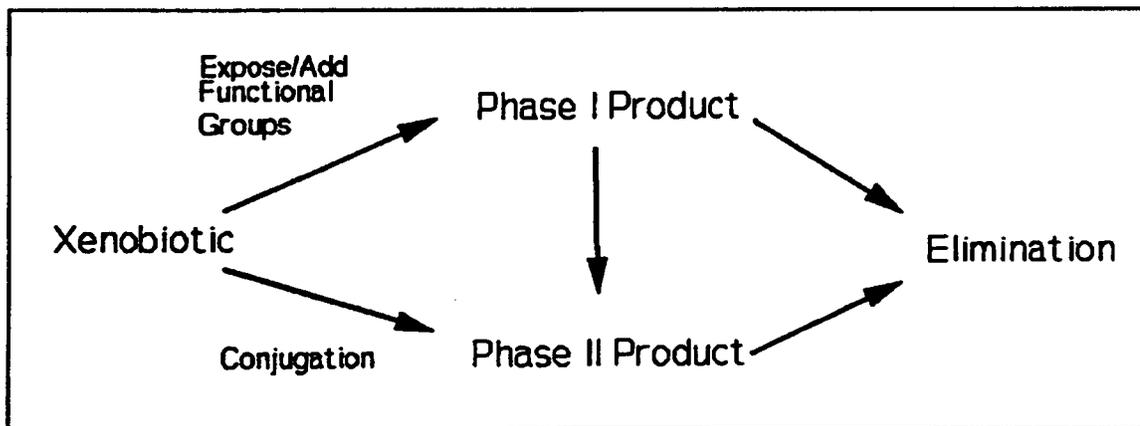


Figure 1. Schematic of phase I and II metabolism

with excess xenobiotic or phase I product and cannot metabolize the reactive compounds fast enough to prevent DNA damage. The endogenous phase II conjugating molecule may also be depleted.

Heat Shock Proteins (Refs. 91-93)

Heat shock proteins (HSP) are a family of proteins found in the cytosol of almost all types of cells. Levels of HSP are increased upon exposure to elevated temperatures and other cellular stresses. Little is known of the function of HSP, but it is generally believed that these proteins enable cells to have an increased thermotolerance, help stabilize other proteins, and aid in translocation of macromolecules within the cell. Elevated levels of HSP have been suggested as a biomarker of exposure to environmental contaminants. HSPs are detected using electrophoresis or column chromatography.

Cytotoxicity (Refs. 94-100)

The effect of DNA damage depends on the function of the area of the DNA to which the damage occurred and whether the damage was repaired. Damage to nonsense regions of DNA (regions that do not code for a particular protein) will usually not have an effect on the function of the cell, but may alter replication of DNA during cell division. In this case, daughter cells may not be formed, leading to the death of the parent cell. Damage to genes that code for functional proteins (enzymes) or structural proteins necessary for cell viability would also cause cell death. Since DNA damage occurs in a seemingly random manner to cells within an organ, not all of the cells with DNA damage would be expected to die. Some cells with DNA damage may be initiated while others die. Thus, cytotoxicity (cellular injury or death) may be used as an indirect determinant of genotoxicant exposure.

Cytotoxicity may be evaluated in several ways. Upon cell damage or death, most cells release enzymes or other proteins that can be used as markers of cytotoxicity. Alanine aminotransferase (ALT) is such an enzyme that is indicative of *in vivo* liver cell (hepatocyte) injury or death. Serum levels of ALT increase dramatically upon low-level hepatotoxicant exposure. *In vitro* cytotoxicity may be measured with dyes such as trypan blue, neutral red, ethidium homodimer-1, and calcein AM.

Ornithine Decarboxylase (Refs. 101-109)

Ornithine decarboxylase (ODC) is an enzyme indicative of cellular proliferation, signaling possible exposure to a cancer promoter. ODC removes a carboxyl (-CO₂H) group from ornithine, a derivative of the amino acid arginine, to form putrescine, the initial product in the polyamine biosynthetic pathway. Polyamines are normally present at very low levels in quiescent cells, but are elevated many-fold during periods of active cell division. To assay for ODC activity, livers from exposed organisms are isolated and prepared using ultracentrifugation. Ornithine having a radiolabeled carboxyl group is

incubated with the enzyme preparation, and the metabolized radiolabeled carboxyl group, which is liberated as a gas, is trapped and quantitated.

Oxidative Stress (Refs. 110-118)

While oxygen (O_2) is essential for all multicellular organisms, some forms of oxygen produced during the metabolism of oxygen, that is, superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH\cdot$), and hydrogen peroxide (H_2O_2), are highly reactive. These oxyradicals can upset the reduction-oxidation (redox) potential of the cell, leading to a highly oxidizing environment within the cell. The redox potential of cells is normally tightly controlled by cellular antioxidant defense mechanisms. These mechanisms include the enzymes superoxide dismutase, catalase, and glutathione peroxidase, as well as vitamins (β -carotene, α -tocopherol, retinoic acid, and ascorbic acid), and the tripeptide glutathione. Cellular antioxidant defense mechanisms can be overwhelmed by xenobiotic chemicals that induce oxidative stress, exemplified by paraquat and quinones. Consequently, genomic function can be impaired due to alterations in DNA, such as the formation of 8-hydroxydeoxyguanosine adducts. Enzyme function may also be inactivated and cell membranes may be disrupted due to protein and lipid oxidation, ultimately resulting in cell death.

Oxidative stress is evaluated by measuring either the oxygen radicals themselves, oxyadducts such as 8-hydroxydeoxyguanosine, or the induction of oxidative stress defense mechanisms such as glutathione.



Environmental Effects of Dredging Technical Notes



Methods for the Assessment of the Genotoxic Effects of Environmental Contaminants; Cellular and Organ/Organism Effects

Purpose

This technical note is the second in a series of three that outline and describe the principal methods that have been developed to test the potential of environmental contaminants for causing mutagenic, carcinogenic, and teratogenic effects. The first in this series (EEDP-04-24) describes methods used to discern genotoxic effects at the subcellular level, while this technical note describes methods used to discern genotoxic effects at the cellular and organ/organism level. Recent literature citations for each topic are listed in the third technical note (EEDP-04-26) to assist readers in locating source information. A glossary of terms is also provided in Technical Note EEDP-04-26.

The information in these technical notes is intended to provide Corps of Engineers personnel with a working knowledge of the terminology and conceptual basis of genotoxicity testing. To develop an improved understanding of the concepts of genotoxicity, readers are encouraged to review "A Primer in Genotoxicity" (Jarvis, Reilly, and Lutz 1993), presented in Volume D-93-3 of the *Environmental Effects of Dredging* information exchange bulletin.

Additional Information

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Cytogenetics (Refs. 1-10)*

Cytogenetics is the study of genetic damage that is discernible at the cellular level. Cytogenetic procedures often involve isolating cells from exposed animals or using tissue slices from specific organs and analyzing them for gross abnormalities. Commonly used cytogenetic assays are the micronucleus assay, sister chromatid exchanges, and chromosome aberrations.

To realize how toxicants can affect cells, it is essential to have a basic understanding of cell division. Mitosis is the cell division of somatic cells, that is, all cells except the sex, or germ, cells. The mitotic cycle has five stages: interphase, prophase, metaphase, anaphase, and telophase. The only visible nuclear changes in interphase are the increasing volumes of the nucleus and nucleolus. In prophase, nuclear division and contraction by coiling of the chromosomes has started. Individual chromosomes can be distinguished and are seen to be double. By the end of this phase, the nucleoli shrink and disappear. In prometaphase, the spindle is organized and some fibers run from each pole to the centromeres of each chromosome. In the short time of metaphase, the centromeres are usually aligned along the equator, and daughter centromeres are still attached to each other. The coiling of chromatids is completed. In the anaphase stage, the division of centromeres is completed and the centromeres of each chromosome pair separate. Movement ceases when all the centromeres are aggregated closely about the poles. In telophase, the nuclear envelope reforms as do the nucleoli. Telophase is completed when the nuclear membrane is finished and the nucleoli have reached full size, creating two complete cells.

Micronucleus Assay (Refs. 119-132)

Micronuclei are small, secondary nuclei formed during telophase in cell division. They develop from chromatin (DNA and associated nucleoproteins) lagged in anaphase resulting from chromosome breakage or a malfunction of the spindle apparatus. To assay for micronuclei, cells either from tissues of exposed animals or grown in culture and exposed in vitro are isolated and fixed on microscope slides. The cells are stained with Giemsa, which allows visualization of nuclei using microscopy. The percentage of micronuclei cells in the exposed animal is compared to that of a control animal. An increase in the frequency of micronucleated cells present in a tissue is an index of chromosome damage associated with exposure to a genotoxic agent.

* Refer to bibliographic citations 1 through 10 in *Environmental Effects of Dredging* Technical Note EEDP-04-26.

Sister Chromatid Exchanges (Refs. 133-143)

During cell division, the chromosomes in the parent cell divide into two chromatids and replicate themselves. Each of the two daughter cells formed receives one of the parent chromatids in addition to the newly created, replicate (sister) chromatid. A sister chromatid exchange (SCE) is a mutagenic event in which an exchange of chromatin occurs between two sister chromatids at the same locus, caused by a break in both of the DNA strands. An *in vitro* SCE assay is typically performed using Chinese hamster ovary (CHO) cells since they contain a small number (21) of relatively large chromosomes.

In the SCE assay, CHO cells are incubated with the test chemical for 2 hr, and the exposure is terminated by changing the cell growth medium. The treated cells are then incubated for 24 to 30 hr with 5-bromo-2'-deoxyuridine, a fluorescent analog of deoxyuridine, a precursor of one of the four DNA bases, deoxythymidine. During this incubation time, the fluorescent base is incorporated into the daughter chromatid. The cells are fixed onto microscope slides and stained with Giemsa and Hoechst 33258, which allows visualization of the chromosomes upon exposure to ultraviolet light. Figure 1A illustrates a typical fluorescence pattern of control cells, with the daughter chromatid containing almost all of the incorporated fluorescent base (dark chromatids). Note that background SCEs do occur. Figure 1B illustrates a typical fluorescence pattern of exposed cells.

Chromosome Aberrations (Refs. 144-155)

Chromosome aberrations are the formation of chromosomes that are different from the original chromosomes. The expression of chromosome damage is usually dependent on cells performing DNA replication and nuclear division. These changes can occur structurally and numerically. Structurally, a normal chromosome consists of a centromere that determines the morphology of the

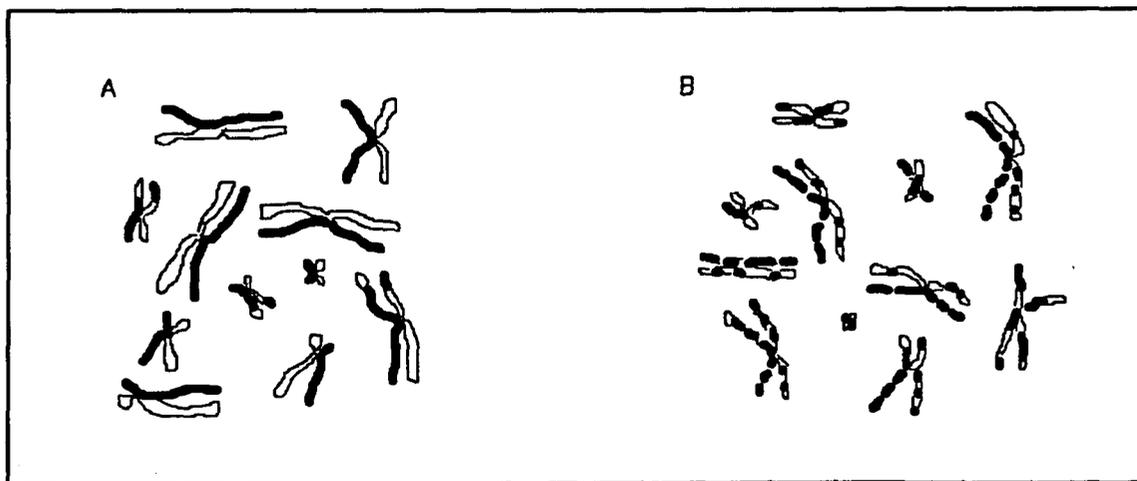


Figure 1. Sister chromatid exchanges in control cells (A) and cells treated with a mutagen (B)

chromosome and two chromatids, each composed of two complementary DNA strands. There are three distinct normal shapes of chromosomes: metacentric (V-shaped), submetacentric (J-shaped), or acrocentric (I-shaped).

Structural aberrations of chromosomes include chromosome breakage, inversion, and translocations. Chromosomal breakage includes single chromosome breaks with fragments, double breaks with deletion, duplication, or ring chromosome formation as shown in Figure 2. Inversion occurs when breaks in the chromosome swing around 180 deg and rejoin at the ends, and the whole segment of the chromosome lies in inverse genetic order.

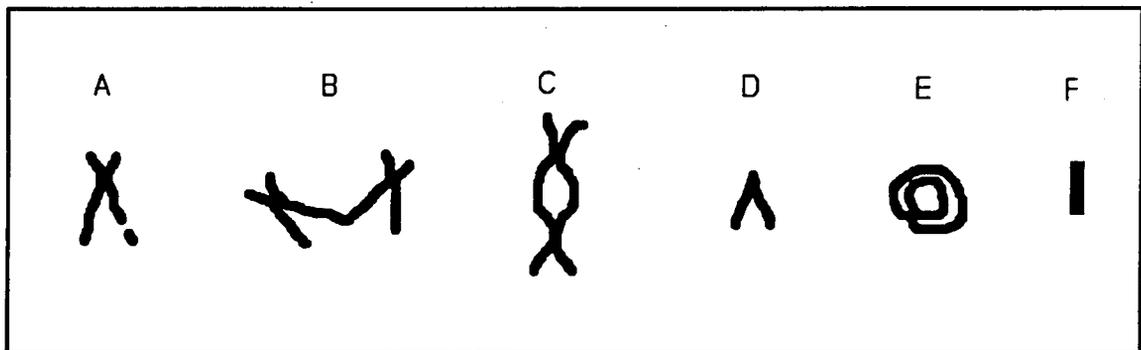


Figure 2. Types of chromosome aberrations: (A) chromatid deletion, (B) triradial chromatid exchange, (C) dicentric chromosome, (D) acentric fragment, (E) centric ring, and (F) small interstitial deletion

Sometimes in cell division, chromosomes have an abnormal number of centromeres. One sister chromosome will have two centromeres and is called dicentric while the other chromosome does not have a centromere and is termed acentric (also illustrated in Figure 2). Dicentric and acentric chromosomes are considered unstable because they are frequently lost from cells when two centromeres proceed to the opposite poles in cell division.

Another type of chromosomal aberration is change in the chromosome number. Aneuploidy is the gain or loss of chromosomes in which the chromosome number differs from the normal haploid (n) or diploid ($2n$) chromosome number. Aneuploidy usually occurs as a consequence of errors in nuclear division. Nondisjunction contributes to aneuploidy and is a chromosome loss that occurs because of anaphase lagging. Also with anaphase lagging, one or more chromosomes can be lost because some of the chromosomes may fail to move to the poles of the cell during anaphase and will thus be left out of the nucleus when the nuclear membrane is reformed. Nonspecified metaphases occur when the morphology of the chromosomes is not well defined.

Polyploidy occurs when a diploid gamete unites with a normal haploid gamete during fertilization and a triploid ($3n$) zygote results.

Histopathology (Refs. 156-179)

Histopathology offers biomarkers that integrate the adverse biochemical and cellular effects of xenobiotics to indicate whether target organs/organisms have been compromised. Biochemical alterations can cumulate into cellular alterations which, in turn, can cumulate into histopathological alterations (lesions) of organ systems. Thus, histopathology provides a means of monitoring the actual health of aquatic species, rather than using biochemical and cellular alterations to predict possible adverse effects.

Drawbacks of histopathology include the inability to ascertain specific etiologic agents and the uncertainty of distinguishing lesions caused by infectious disease, normal physiologic variation, or natural toxins from lesions caused by anthropogenic chemicals. Also, the organism must be sacrificed for histological examination, eliminating the possibility of time-sequenced examination of the same organism.

Histopathology involves sacrificing the subject organism and isolating the organ to be examined. The tissue is preserved with a fixative (formalin, for example) and prepared for sectioning. Preparation for tissue sectioning usually entails dehydrating the tissue by placing it in a series of alcohols and solvents and infiltrating the tissue with paraffin to fill in the dehydrated spaces of the tissue. The tissue is then embedded in paraffin and sectioned using a microtome, which is capable of slicing sections 1 μm thick. The thin tissue sections are mounted onto microscope slides and stained for microscopic examination. The most common types of damage that are looked for are described below.

While other organs experience lesions and are examined histopathologically, the liver is the most-used organ for such studies. The liver is highly perfused with blood, receiving the highest percentage of cardiac output of all organ systems (~ 27 percent, depending on species). Chemicals absorbed from the diet (nutrients and xenobiotics) are transported directly to the liver for "processing" before they are distributed to other parts of the body. The liver is rich in xenobiotic metabolism enzymes, and most phase I and II metabolism occurs there. If reactive metabolites are formed, most react in the liver, the site of metabolism. Therefore, the liver is the most probable initial site of action for most toxicants.

Hepatocellular Necrosis

Hepatocellular necrosis is, by definition, death of liver cells, or hepatocytes, and usually occurs as the result of a sudden cessation of blood flow or damage by toxic agents. Necrotic changes often are focal or multifocal, in that they occur in localized areas within the organ. The liver consists of approximately 40 different cell types, which vary widely in their purpose. Foci of necrotic cells may be observed in, for example, cells with high cytochrome P450 content, indicating possible exposure to xenobiotics. Toxicant-related hepatic

necrosis must be differentiated from necrosis due to postmortem changes. As such, stringent sampling and tissue fixation protocols are required.

Hyperplasia of Regeneration

After hepatocellular necrosis occurs, if the organism survives, the remaining hepatocytes undergo hyperplasia of regeneration, replacing necrotic cells. The replacement cells are smaller, irregularly shaped cells which form islands at the foci of the necrotic cells. These areas of hyperplasia of regeneration are used as indicators of prior hepatocellular necrosis.

Hepatocytomegaly

Hepatocytomegaly is an enlargement of the hepatocytes and is generally classified into three types: hepatocellular hypertrophy, megalocytosis, and hepatocellular vacuolation. Hepatocellular hypertrophy is an enlargement of cellular diameter without accompanying nuclear changes, leading to a net gain in the dry mass of the liver. A common cause of hepatocellular hypertrophy is proliferation of endoplasmic reticulum, indicating induction of cytochrome P450, that is, exposure to cytochrome P450-inducing compounds. Megalocytosis is characterized by enlargement of both the cell and the nucleus, and hepatocellular vacuolation is characterized by vacuolation, or formation of pockets of fluid within the hepatocytes. Little is known about the mechanism of the latter two types of hepatocytomegaly, but all three types are associated with exposure to genotoxic contaminants.

Foci of Cellular Alteration

Foci of cellular alteration, also known as staining or tinctorial changes, become apparent upon staining of liver sections. The conventional stains used in histopathology, hematoxylin and eosin, stain hepatocytes different colors and different intensities of color depending on cellular content. Hematoxylin is a basic dye, and cells that it stains are termed basophilic. Basophilic cells are depleted of glycogen and have increased levels of cytoplasmic RNA, both indicating protein synthesis. Eosin is an acidic stain that stains eosinophilic cells, which are generally hypertrophic and have a reduced glycogen content. Clear cells are cells that stain with neither hematoxylin nor eosin and are glycogen rich. Foci, or localized areas of similarly staining cells, can indicate areas of toxicant effect.

Foci of Enzyme Alteration

Foci of enzyme alteration is a newer technique based upon foci of cellular alteration. This technique uses a different tissue sectioning method: cryostat-ically sectioned tissue slices. Rather than paraffin-embedding the liver, it is placed in a mold and embedded in a glycol/resin compound that hardens to about the same consistency as paraffin upon freezing. The frozen tissue is then sectioned using a cryostat, which is basically a microtome in a freezer.

The frozen tissue sections are mounted onto microscope slides and still maintain their enzyme functions.

Determination of foci of enzyme alteration may be accomplished using histochemical, immunohistochemical, and in situ hybridization techniques. Histochemical techniques involve flooding the tissue section with a particular substrate for the enzyme of interest that is an irreversible inhibitor of the enzyme; that is, the substrate covalently binds to the enzyme. A radiolabeled, colored, or fluorescent substrate is used, and the foci of enzyme alteration (for example, induction of a particular enzyme) can be noted with light microscopic autoradiography, light microscopy, and fluorescence microscopy, respectively. Light microscopic autoradiography involves dipping the slides into a special photographic emulsion in the dark and developing the slides with photographic fixer and developer, which stains the bound radiolabeled substrate with silver grains. Immunohistochemical techniques utilize the antigen-antibody principle, in that an antibody to a specific enzyme is constructed and incubated with the tissue section. The antibody binds to the enzyme/antigen and is visualized using the same means as with the histochemical techniques. Both histochemical and immunohistochemical techniques assay for enzymes, with the immunohistochemical techniques being more specific for a particular enzyme.

In situ hybridization examines enzyme alteration at the molecular level rather than at the protein level. If a protein/enzyme is induced in response to toxicant exposure, messenger RNA (mRNA) specific for that protein increases within the cell, which then is translated into the protein. A probe for that particular mRNA, which is a length of complementary RNA (cRNA) that will hybridize (or noncovalently bind) to the mRNA, is used that is radiolabeled or fluorescent labeled. The probe is incubated with the tissue section for hybridization and visualized using the appropriate technique (light microscopic autoradiography or fluorescence). Foci of enzyme alteration, like foci of cellular alteration, can indicate genotoxicant exposure.

Neoplasms

Neoplasms are cancers or tumors and are one of the least-desired contaminant effects in aquatic populations. Therefore, neoplasms are a signal of definite genotoxic contamination, while the biomarkers discussed earlier are signals of potential genotoxic contamination. Several types of hepatic neoplasms are associated with genotoxic contaminant exposure and are named based on the type of tissue in which they occur. Hepatic adenomas, hepatocellular carcinomas, cholangiomas, and mixed hepato-cholangiocellular carcinomas are the most important such neoplasms. Neoplasms are diagnosed either through histopathological methods or by gross autopsy.

Nonhepatic Tissues

Tissues other than the liver are also used histopathologically to assess genotoxicant exposure. Ovary and sperm morphology, skin neoplasms, and spleen,

kidney, and brain histopathologies are also used to some degree, as these are sites of action of certain specific toxicants.

Developmental Abnormalities (Refs. 180-201)

Embryonic development can be considered a weak link in the life cycle of an organism because, during this period, distinctive cellular and molecular processes operate to form a complex multicellular organism from an embryo. These processes can be easily disturbed by many chemicals. Developmental toxicants exert their effects on embryos at concentrations lower than those required to affect adults or cause general cellular toxicity.

One type of assay used to assess developmental effects in aquatic organisms is early life stages (ELS) testing. ELS involves exposing organisms at the time of fertilization until some later time period and observing for hatching success, survival and growth of larvae, and often, deformities of larvae. Organisms commonly used for ELS testing include Japanese medaka, rainbow trout, and the South American clawed frog (*Xenopus laevis*) (the FETAX, frog embryo teratogenesis assay-*Xenopus*).



Environmental Effects of Dredging Technical Notes



Methods for the Assessment of the Genotoxic Effects of Environmental Contaminants; Glossary and References

Purpose

This technical note is the third in a series of three that outline and describe the principal methods that have been developed to test the potential of environmental contaminants to cause mutagenic, carcinogenic, and teratogenic effects. The first in this series (EEDP-04-24) describes methods used to discern genotoxic effects at the subcellular level, while the second (EEDP-04-25) describes methods used to discern genotoxic effects at the cellular and organ/organism level.

Recent literature citations for each topic referenced in this series of technical notes are provided in this technical note, in addition to a glossary of terms. The information in these technical notes is intended to provide Corps of Engineers personnel with a working knowledge of the terminology and conceptual basis of genotoxicity testing. To develop an improved understanding of the concepts of genotoxicity, readers are encouraged to review "A Primer in Genotoxicity" (Jarvis, Reilly, and Lutz 1993), presented in Volume D-93-3 of the *Environmental Effects of Dredging* information exchange bulletin.

Additional Information

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Glossary

Adduct - a chemically modified macromolecule. An adduct is formed when a compound covalently binds to DNA, hemoglobin, bile, etc.

Amino acid - an organic acid having the general structure of $\text{HO}_2\text{C}-\text{CHR}-\text{NH}_2$ linked together polymerically to form proteins. The R group determines the specific amino acid. Amino acids are obtained from the diet (essential amino acids) or are synthesized by the body from essential amino acids (nonessential amino acids).

Analog - a compound structurally similar to another.

Antigen-antibody reaction - an antigen is a foreign compound that enters the body. In response to an antigen, particular cells in the body synthesize antibodies, or proteins that bind to the antigen and are highly specific for the antigen. The antigen-antibody complex is recognized by other types of cells in the body, which engulf the antigen-antibody complex for removal from the body. This immune-response principle the body uses to fight infection has been applied to the detection of particular compounds (antigens) of interest. Antibodies to the compound or enzyme of interest are synthesized using biotechnology techniques. The antibodies are labeled with a fluorescent tag that allows visualization using fluorescence techniques.

Autoradiography - a method of visualizing distinct areas of radioactivity in a sample using photographic techniques. Radioactive compounds emit energy in the form of gamma rays or alpha or beta particles, depending on the type of isotope. This energy will expose photographic film in the same manner as visible light, giving a "picture" of the location of radioactivity in a sample.

Bioluminescence - a biochemical reaction occurring in an organism that results in the formation of light energy. An example is the light produced by a firefly.

Biomarker - generally, some biological event that can be used to signal the exposure of an organism to a particular contaminant.

Carcinogen - a compound shown to cause the formation of cancer in an organism.

Cardiac output - blood flow from the heart.

Chromosome - the condensed form of DNA and its associated proteins visible during cell division.

Complementary RNA or DNA (cRNA, cDNA) - a strand of RNA or DNA that is composed of bases complementary to a particular segment of RNA or DNA. RNA is composed of four bases: guanine (G), which is complementary to cytosine (C), and adenine (A), which is complementary to uracil (U). DNA is composed of these same bases, except thymine (T) is substituted for uracil. Complementary bases noncovalently bind (hybridize) with each other. A segment of cDNA for a piece of DNA whose sequence is CCGATAAGT would be GGCTATTCA. cDNA and cRNA are often used as probes.

Conjugation - the covalent bonding of an endogenous molecule to a xenobiotic molecule to facilitate excretion of the xenobiotic.

Covalent bonding - an interaction of two or more separate molecules whereby they become one distinct molecule. For example, two hydrogen and one oxygen atoms may covalently bond to form water.

Cryostatic - techniques that are performed at freezing temperatures.

Cytochrome P450 - a family of enzymes located primarily in the liver that normally function in steroid metabolism, but which also metabolize xenobiotic compounds.

Cytosol - the aqueous portion of a cell and the components dissolved therein. The cytosol is isolated from tissues using ultracentrifugation.

DNA - deoxyribonucleic acid, an extremely long molecule composed of four nucleotides (adenine, thymine, cytosine, guanosine) which contains the genetic makeup of an organism.

Electrophoresis - a method used to separate large electrically charged molecules such as DNA, RNA, or proteins. Electrophoresis uses direct electrical current to cause the charged molecules to migrate through a gel toward the oppositely charged pole of the apparatus.

Endoplasmic reticulum - a flat, membranous, netlike system within the cytoplasm of a cell that, among other functions, contains cytochrome P450.

EROD induction assay - ethoxyresorufin-O-deethylase, or EROD, is a particular cytochrome P450 enzyme that is normally present in the liver in very small quantities, but is induced upon exposure to planar aromatic compounds such as dioxins, furans, and PCBs. For the EROD induction assay, liver hepatoma (cancer) cells grown in culture are dosed with sample extracts and analyzed for EROD activity using a fluorometer. This assay detects picogram (parts per trillion) quantities of 2,3,7,8-TCDD, the most potent EROD inducer, rivaling gas chromatography/mass spectrometry in sensitivity.

Etiologic agent - a compound that can be shown to be the cause for some effect.

Eukaryote - a cell that comprises a multicellular organism. Eukaryotic cells are much more complex than prokaryotic cells, containing more subcellular components.

Fluorescence - the emission of light of a particular wavelength by a compound after absorption of light of another wavelength. Fluorescence is the basis for detection of many compounds and is extremely sensitive and somewhat specific.

Fluorometer - an instrument used to measure fluorescence.

Genome - the DNA of a cell.

Glucose - a simple sugar (monosaccharide) that is utilized by cells for energy.

Glutathione - a tripeptide, γ -glutamylcysteinylglycine, that is found in virtually all species. Glutathione is extremely important in that it helps regulate the reduction-oxidation potential of the cell, acts as an amino acid transport system for the body, and functions in phase II metabolism.

Glycogen - the storage form of glucose. After periods of glucose intake (eating) when blood glucose levels are high, the body stores excess glucose by linking, or polymerizing, the glucose molecules together into a branched chain configuration making glycogen. Glycogen is stored in the liver and muscle.

Hybridize - noncovalent bonding of complementary segments of nucleic acids.

Hyperplasia - an excessive proliferation of normal cells.

Hypertrophy - an enlargement of cellular diameter without accompanying nuclear changes.

Induction - production of a particular protein in response to some stimulus.

Initiation - conversion of a normal cell to a cancerous cell. Initiation is an irreversible change involving the interaction of a carcinogen with DNA, priming the cell for cancer development via promotion.

Lagging - a term used to describe the leaving behind of part of a chromosome during the migration of chromosomes in anaphase during mitosis.

Luminometer - an instrument used to measure bioluminescence.

Macromolecules - large molecules that comprise a cell. Macromolecules include DNA, RNA, and proteins.

Messenger RNA - a strand of RNA that is complementary to a particular segment of DNA (gene) and acts as a template for the translation (production) of a particular protein.

Mutagen - a compound that can cause a mutation, or a change in a specific DNA nucleotide, for example, adduct formation.

Nucleoside - a building block of DNA and RNA. A nucleoside is one of the five nitrogen bases (adenine, guanine, uracil, cytosine, and thymine) linked to a sugar compound called ribose (for RNA) or deoxyribose (for DNA).

Nucleotide - a nucleotide is a nucleoside with a phosphate group attached. Nucleosides must be converted to nucleotides before they can be incorporated into DNA or RNA.

Phase I metabolism - the metabolism of xenobiotic compounds by enzymes which include cytochrome P450. Phase I metabolism usually results in the addition or exposure of a polar functional group, for example, an -OH group, on the xenobiotic. Phase I metabolism readies the xenobiotic for urinary excretion or phase II metabolism.

Phase II metabolism - the metabolism of xenobiotic compounds whereby an endogenous molecule is conjugated with a xenobiotic. Phase II metabolism readies the xenobiotic for biliary excretion.

Probe - a relatively short strand of RNA or DNA that is complementary to a particular gene of interest and is labeled with a fluorescent or radioactive tag. The probe is incubated with isolated DNA or RNA from an organ and hybridizes with the gene of interest. The gene may then be visualized using fluorescent microscopy or autoradiography.

Prokaryote - a bacterial cell. Prokaryotic cells are much simpler than eukaryotic cells, lacking many of the subcellular structures of the eukaryotic cell. One-celled organisms are prokaryotic.

Promotion - the process by which a chemical facilitates the growth and development of initiated cells into a tumor. Promoters do not interact directly with DNA, but generally stimulate an increase in DNA synthesis and/or cell replication in the target cells.

Promutagen - a compound which, when metabolized, is converted into a mutagen.

Teratogen - a compound causing defects in reproduction, resulting either in reduced productivity due to fetal mortality or in the birth of offspring with physical, mental, behavioral, or developmental defects.

Translation - the biosynthesis of amino acids.

Ultracentrifugation - a technique involving centrifugation at extremely high speeds, up to 250,000 times the force of gravity, used to isolate cellular components.

Xenobiotic - a compound foreign to the body. Examples of xenobiotics are pesticides, PAHs, and dioxins.

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