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Monitoring Genetic Damage to Ecosystems from Hazardous Waste

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MONITORING GENETIC DAMAGE TO ECOSYSTEMS FROM HAZARDOUS WASTE

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ABSTRACT

Applications of ecological toxicity testing to hazardous waste management have increased dramatically over the last few years, resulting in a greater awareness of the need for improved biomonitoring techniques. Our laboratory is developing advanced techniques to assess the genotoxic effects of environmental contamination on ecosystems. We have developed a novel mutagenesis assay using the nematode *Caenorhabditis elegans*, which is potentially applicable for multimedia studies in soil, sediment, and water. In addition, we are conducting validation studies of a previously developed anaphase aberration test that utilizes sea urchin embryos. Other related efforts include field validation studies of the new tests, evaluation of their potential ecological relevance, and analysis of their sensitivity relative to that of existing toxicity tests that assess only lethal effects, rather than genetic damage.

INTRODUCTION

In ecological risk assessment, toxicity testing is extensively used to evaluate potential ecological hazards associated with waste management practices. Toxicity tests are used in such applications as pre- and post-disposal monitoring, evaluation of the progress of remediation or treatment activities, design of experimental treatment systems, identification of causes and sources of toxicity, and determinations of the degree and extent of contamination at waste sites, to "map" areas that should be prioritized for remediation. Toxicity tests are most useful in evaluation of the effects of mixed wastes, which contain many chemicals in combination. This is because analyses of chemical concentrations alone cannot provide adequate predictions of the effects of even a few chemicals acting together. As biologists learn to understand engineering and environmental management concerns, the number of applications of toxicity testing have expanded rapidly. Simultaneously, engineers have become more accepting of the potential value and the potential precision of biological measurements (Anderson and Norberg, 1991).

Consequently, the applications of toxicity testing have increased in surface water management as well as in hazardous waste management, but the development of new

toxicity tests has not kept pace with this increasing demand. Limitations of the existing tests, which hamper more widespread application, are that few tests exist for multimedia evaluations (e.g. tests for soil, sediment and sludge are limited) and that few tests exist that quantify sublethal effects of contaminant exposure. As a result, management decisions are mainly based on whether an animal lives or dies; other effects such as genetic damage or alterations in reproductive success are rarely measured.

We are interested in increasing the breadth of short-term toxicity test methods to include detection of genetic and reproductive effects. In more general terms, we study the question "How can genetic toxicology be incorporated into ecological risk assessment?"

This paper describes our integrated program to develop the potential applications of genetic toxicology in ecological risk assessment. Our approach and recent progress are discussed in the context of the existing literature and the potential for providing management insights.

BACKGROUND

A stark contrast exists between the status of genetic toxicology in ecological risk assessment and the status of genetic toxicology in human health risk assessment. In ecotoxicology, almost nothing has been done to prioritize potential approaches to assessing the effects of genotoxic substances or to distinguish the ways in which genotoxic effects may differ from the effects of other toxic substances. In general terms, five approaches exist to evaluating genotoxic effects in ecosystems.

The first general approach has been the development of a modest number of genotoxicity bioassays, mainly using plants and aquatic animals (Kligerman, 1982 and Landolt and Kocan, 1983 for brief reviews). Endpoints evaluated have varied, but typically, effects such as micronuclei (e.g. Jaylet et al., 1986 and Krauter et al., 1987), anaphase aberrations (Hose and Puffer, 1983), sister chromatid exchanges (e.g. Pesch et al., 1981 and Harrison and Jones, 1982) and chromosomal aberrations (e.g. Anderson et al., 1990) have been evaluated. With the exception of anaphase aberration assays and micronuclei assays, the methods have proved excessively laborious in organisms with low mitotic indices. Consequently, these methods have not been applied in any meaningful manner to field situations or to a broad range of environmental chemicals in the laboratory. Anaphase aberration and micronuclei tests have been used in field assessments, but (with the possible exception of a *Tradescentia* micronucleus assay) these methods can only be applied on a seasonal basis because the organisms to be studied are not available year round. In addition, micronuclei assays have been applied without prior characterization of control frequencies that vary among lifestages, with temperature and other variables that may differ between experimental and reference sites in the field. In summary, a small collection of bioassays exist, and a few of these warrant further characterization. However, no animal tests have been developed that can be used in a range of media (aqueous and solid) or throughout the year. Only one of the tests can

be used to evaluate multiple generation laboratory exposures (Harrison and Anderson, 1988, 1989).

The second general approach is the use of tests originally employed in human health-based studies. These include, but are not limited to, the Ames test (e.g. Stahl, 1991 for review, de Raat et al., 1985, McDaniels et al., 1990, Blevins and Brennan, 1990), CHO cells *in vitro* (Waters et al., 1987), and the SOS chromotest (McDaniels et al., 1990). This approach is appropriate for preliminary screening-level assessments, provided that varied methods of metabolic activation are considered. Various investigators have evaluated methods of activation by aquatic and terrestrial plants (e.g. Schoeny et al., 1985 and Harwood et al., 1989) as well as by fish microsomal preparations (e.g. Coulombe et al., 1982, Goubaitis et al., 1986, and Kurelec and Krca, 1987). The principle limitations of this approach are that long term effects cannot be studied, that environmental samples usually cannot be tested without modification, that field exposures cannot be simulated, and most importantly, that no research has been conducted to determine how well these tests predict responses in varied organisms in the environment.

The third general approach has been to survey for cancer in fish species at contaminated sites and in laboratory exposures using standard chemicals (Couch and Harshbarger, 1985). This approach is also valuable but has limited predictive capability; and typically, large volumes of chemical waste are generated in the conduct of long term exposures using fish species. Additionally, many types of organisms have relatively short lifespans and do not get cancer.

The fourth general approach has been to assay for biochemical measures of putative DNA damage such as DNA single strand breaks and DNA adducts in tissues of varied aquatic organisms (e.g. Shugart, 1988 and Varanasi et al., 1989). This approach is primarily useful as a measure of exposure to genotoxic substances; however, it is difficult to extrapolate from the responses observed to significant ecological effects. Moreover, natural substances present in unpolluted environments may cause adducts that are difficult to distinguish from those caused by anthropogenic contaminants (Kurelec et al., 1989).

The fifth general approach has been to assess dominant and recessive lethal mutations in varied aquatic species (e.g. Harrison and Anderson, 1988). While this approach is relatively difficult in mammals, it is relatively simple in varied invertebrate species with short lifecycles. This area of research can provide information that is relevant to determining the potential ecological significance of exposure to genotoxic substances and the multigeneration effects of chronic exposures. However, field applications are often limited.

Progress in these five areas has helped to shape the field, but, at this time, there is a need to prioritize the most significant gaps in existing knowledge. One of the most critical needs is that short-term animal tests which are applicable year round should be

developed and thoroughly characterized for application in solid and liquid media. Ideally, these tests should be applicable in field and laboratory exposures and generate low volumes of waste. These tests would be used to screen for potential "hotspots" in the field and to provide a preliminary assessment of the effects of substances in the laboratory. Although genotoxic effects of toxic substances in sediment have been evaluated using fish cell lines and embryos of wild caught fish (Landolt and Kocan, 1983, 1984) as well as using microbial tests (Xu et al., 1987), no studies have used soil-dwelling animals, amenable to laboratory culture, to provide more realistic assessments of *in vivo* exposure conditions in an assay that could eventually be standardized for widespread use. Other researchers (Popham and Webster, 1979 and Williams and Dusenberry, 1990) have suggested the use of *C. elegans* in aquatic toxicology, but these articles have been limited in scope and do not offer suggestions relevant to sediment contamination or genotoxicity. Consequently, our research will be the first to evaluate genotoxic effects in *C. elegans* as a measure of sediment and soil contamination. *C. elegans* is a particularly advantageous species for mutagenicity assessments in sediment and soil because it holds great potential for the assessment of mutational spectra, or the identification of chemical-specific DNA lesions.

Another important gap in our ability to predict the effects of genotoxic substances in the environment is the paucity of approaches to assess multigeneration effects of genotoxic substances. Safe exposure levels cannot be predicted for genotoxic substances if the multigeneration effects of DNA damage are not predicted and if the effects of environmentally realistic chronic exposures are unknown. We know of no efforts in ecotoxicology to predict the effects of genotoxic substances that extend beyond one to two generation exposures (Harrison and Anderson, 1988, 1989; and Marshall, 1962). All of these prior studies have utilized ionizing radiation as the genotoxic agent. Moreover, efforts to model the population-level consequences of exposure to genotoxic substances have not been conducted. With some exceptions (e.g., Barnthouse et al., 1987 and Logan, 1986), modelling efforts to date have primarily consisted of life table evaluations for selected species following one to two generation exposures to toxic substances that are not potently genotoxic (e.g. Marshall, 1962; Daniels and Allen, 1981; Gentile et al., 1982).

Finally, there is a need to link experimental research with ecological theory to delineate the unique effects of genotoxic substances. Research in this area could be valuable in predicting life history strategies that may confer sensitivity to genotoxic substances.

The basic premise of the research conducted in our laboratory to date is that by concentrating mainly on effects in gametes we may be able to provide sensitive measures of effect that also can be interpreted in ecological models. Previously, Harrison and Anderson (1988 and 1989) and Anderson and Harrison (1990) have shown that the effects of ionizing radiation are cumulative in oocytes of the polychaete worm, *Neanthes arenaceodentata*, and that chromosomal aberrations were observed in gametes at similar doses as those that cause such responses in mammals. These findings and those of other researchers (e.g., Woodhead, 1976) lead us to believe that the cellular level of sensitivity

to genotoxic substances may have commonalities among many organisms and tissues, and that genotoxic effects may be cumulative in oocytes of other species. If this finding can be generalized to other species and to genotoxic agents other than ionizing radiation, it would imply that species with long and synchronous periods of gametogenesis may be most vulnerable to the effects of genotoxic substances. To determine whether the findings with the polychaete can be generalized to other species and other genotoxic agents, we have elected to begin cumulative exposure studies with the nematode *C. elegans*. In summary, by concentrating on the gametes, which are arguably the most important cells of any organism in the ecological context, we may be able to provide sensitive and significant measures of DNA damage as well as to discover principles that may be broadly applicable among varied species.

Additional important considerations in the application of genetic toxicology to ecological risk assessment include a need for further studies of metabolic activation systems, for prioritization of relevant chemicals for environmental studies and for comparisons of the sensitivity of ecotoxicological assays to other assays used in health assessments. Many of these topics are currently being adressed in our laboratory, and these efforts are described in the following section.

APPROACH

Our principle goal is to advance the application of genetic toxicology in ecological risk assessment. Genetic damage can cause cancer, mutations and multigeneration reproductive impairment in animal populations; yet, existing tools are not sufficient to provide predictive assessments of such damage. In our laboratory, several projects are being conducted to develop approaches for assessing genetic damage in ecosystems and for determining the sensitivity, applicability, and signifcance of the responses obtained. Our general approach is outlined in Figure 1, and the research is detailed below.

In general terms, we have developed a program that spans a continuum (Figure 1) from the development of short-term tests to their field validation and implementation in management. Our efforts have involved studies on a variety of habitats including soil, sediment, wetland, and estuarine areas, and we have coupled our applied research with significant basic research components.

Development and Validation of a Novel Genotoxicity Test Using the Nematode *C. elegans*

The nematode *Caenorhabditis elegans* is potentially an ideal species for environmental genotoxicity assessments. This is the only animal species we know of that can be exposed to air, soil, sediment, and water for which there is sufficient genetic characterization to develop sophisticated mutant screens. Hundreds of mutants have been isolated and characterized. The availability of dozens of mutants that can be

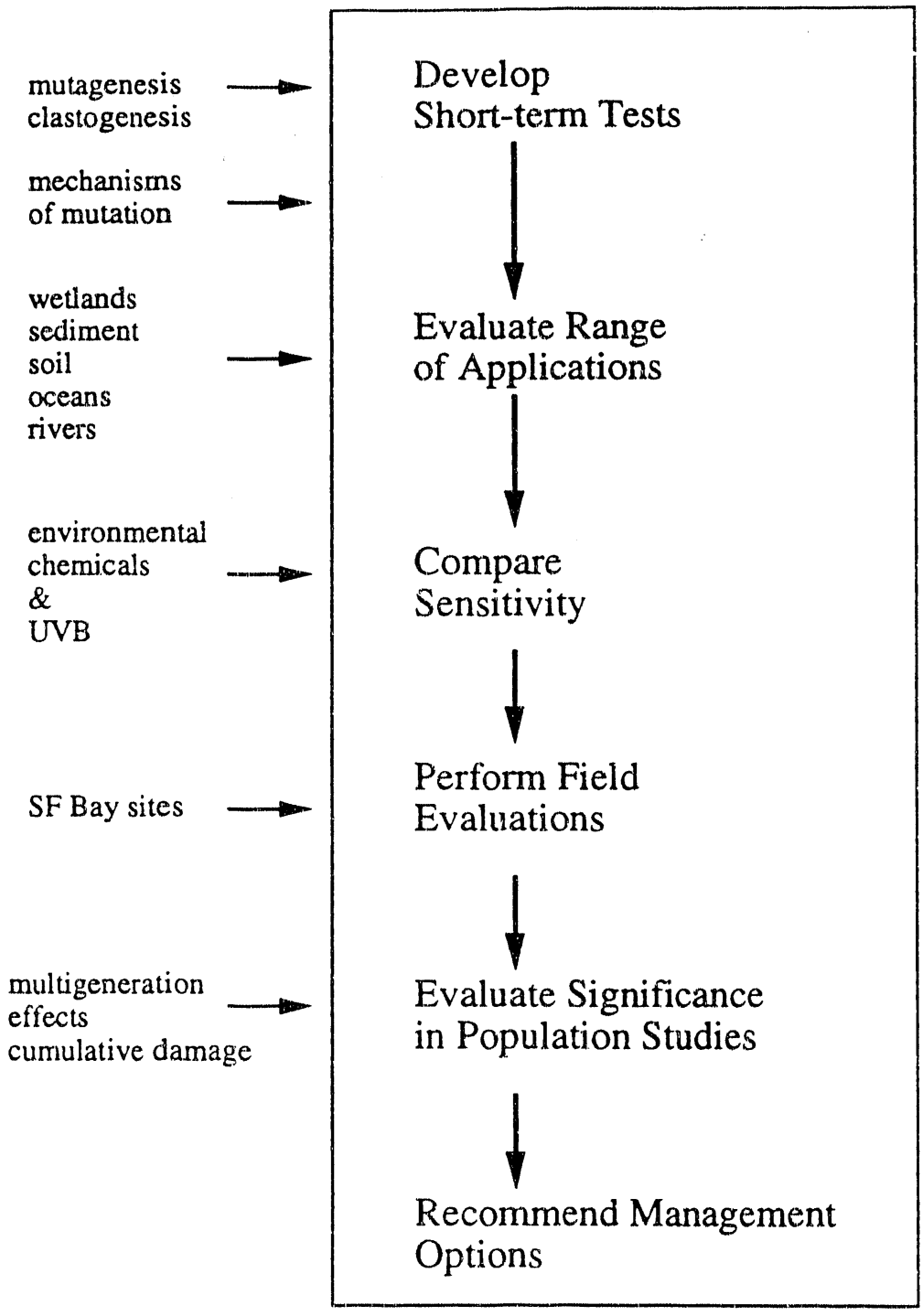

Figure 1. Incorporating Genetic Toxicology Into Ecological Risk Assessment

distinguished under a microscope or using biochemical selection processess, as well as the comparative ease of culturing this species, makes it possible to develop sensitive and practical mutant screens. Additional features of the nematode include: a short generation time of 4-5 days, a hermaphroditic lifestyle which implies that matings do not have to be conducted in multigeneration exposure experiments, extensive knowledge of the timing of development of all of the cells in the worm body (including the gametes), and extensive characterization of the genome of the worm making molecular genetic studies and evaluations of DNA repair mechanisms possible.

Our goal was to build upon basic research in genetics to identify sensitive and practical test systems for environmental mutagenicity assessments. Several genetic screens have been identified in the past, and Rosenbluth et al. (1983) developed a mutagenesis assay utilizing a test strain of *C. elegans(eT1)*. Searching for optimum practical sensitivity in a mutagen test system, we considered many potential test strains, including *eT1*. A number of factors were considered in our initial strain selection. Included were: target size (determined by genetic makeup of the test strain), strain viability, ease of recognition of the induced phenotypic change, which indicates a mutational event, and spontaneous mutation rate. After initial strain selection, the actual logistics of several potential test systems were analyzed. Considerations included the ease of maintenace of the test strain, the number of individuals which could be tested in a given number of days, the induced mutation frequency on exposure to a given dose (45mM) of a known mutagen ethyl methane sulfonate (EMS) compared to the observed spontaneous frequency, and the ease of isolation and confirmation of mutants.

The result of these analyses and preliminary experiments was the selection of two test systems for further study. One was the system of Rosenbluth et al. which involves the induction of recessive lethal mutations in a region of the *C. elegans* genome balanced by the reciprocal translocation *eT1*. Induction of a recessive lethal mutation in parental (PO) gametes results in the total absence of a specific phenotype (DpyUnc) amongst the second generation (F2) progeny. Without the mutational event, the DpyUnc progeny always make up 17% of the total progeny. The second system, we developed utilizing a mutant strain with a defective *unc54* gene. The actual target in this second system is six suppressor (*smg*) genes. The wild-type (non-mutant) *smg* gene product is thought to interact with defective *unc54* gene products, preventing the expression of the *unc54* gene (Hodgkin et al., 1989). The resulting phenotype is a non-motile Unc54. If a mutational event knocks out one of the *smg* genes, the *unc54* gene (though still defective) is able to be expressed, and a motile Smg/Unc54 phenotype results. Thus, mutation of a *smg* gene in the gamete of a *unc54* PO worm results in the appearance of motile *smg/unc54* progeny in the F2 and F3. The screen, therefore, is based on recognizing motile worms against a background of paralyzed worms. The logistics of the assay approach are described in Figure 2.

Dose-response curves for mutant frequency following EMS exposure were completed using the *eT1* and *unc54* mutagen test systems and compared to broodsize dose-response results (Anderson et al., submitted manuscript). Initial doses were selected to compare

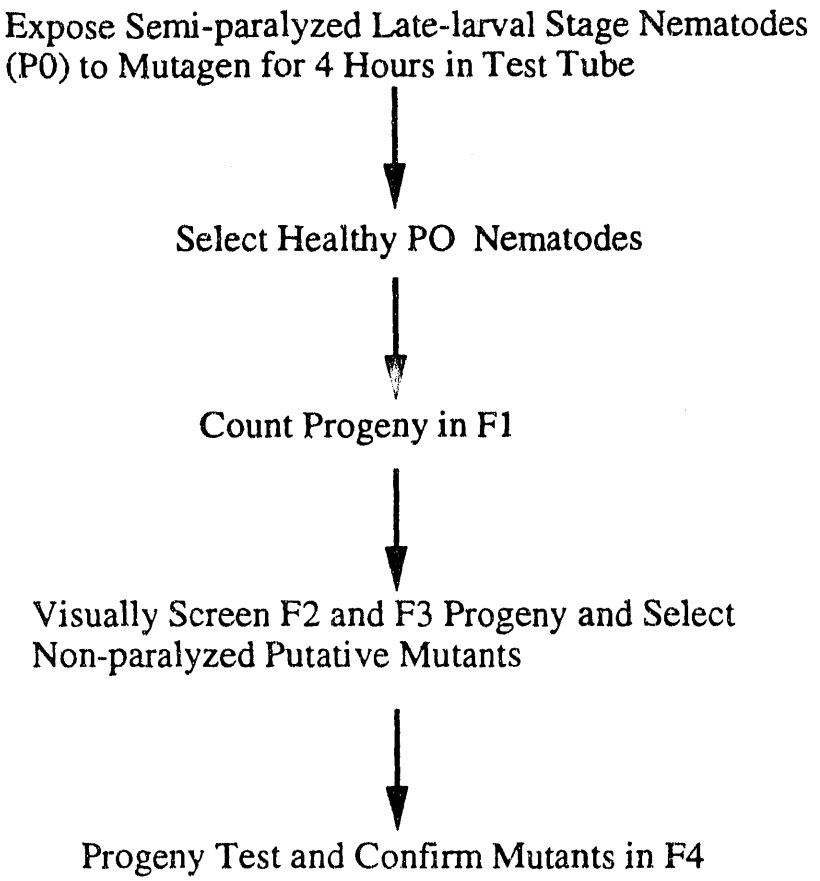

Figure 2. General Procedure For Unc-54 Test Method

reproducibility with published results for the *eT1* system (Rosenbluth et al., 1983). Our subsequent work with both systems concentrated on lower mutagen doses to test the limits of sensitivity. Thus far, significant effects have been detected at the lowest dose tested (0.5mM) using the *unc54* test system, and the sensitivity of the test system is equivalent to that of *eT1*. This also compares favorably with the sensitivity of other sensitive cellular assays. Ellingham et al. (1986) compared the sensitivity of sister chromatid exchange assays in toadfish, eel, human and hamster cells and found that for all of these systems, the lowest doses of EMS at which significant effects were observed ranged from 0.25 to 0.65 mM. Mutagenesis was always a more sensitive response than reproduction (broodsize counts).

Expanding the Applicability of the Nematode Assay and Evaluating its Sensitivity to Genotoxic Substances

We have conducted and planned several experiments to determine the range of applicability of the nematode assay in varied environmental exposures. First, to determine whether the nematode assay can be applied in saltwater or brackish environments as well as in freshwater, we have evaluated the salinity tolerance range of the nematode. Adult L4 stage nematodes were exposed to San Francisco Bay water diluted with a reference mineral water to salinities of 2 to 32 ppt (10 nematodes at each salinity), and the number of F1 progeny were enumerated by microscopic analysis. When the number of F1 progeny in the salinity treatments were compared to the controls, we found that there were no significant differences in any of the salinity treatments as compared to the controls. These data are valuable because the nematode test can now be applied to a broad range of aquatic environments including oceans and estuaries as well as lakes, rivers and streams.

To determine whether the nematode can be used to assess the effects of contaminants in sediment, we have conducted preliminary pore water experiments. Pore water is prepared from sediment cores and is considered to be a representative exposure regime for interstitial species. Pore water samples were obtained from a sampling conducted in May, 1991 at selected reference sites. Working with the San Francisco Bay Regional Water Quality Control Board, we have coordinated our efforts with environmental chemists who collected the pore water and characterized the concentrations of trace metals and organic contaminants in the samples. This effort will allow us to obtain mutation frequencies for pore water that will have been characterized to the fullest extent practicable. This is important because if mutation frequencies increase significantly, we can determine whether the increase is attributable to toxic substances or to other uncharacterized mutagenic substances or exposure conditions. Although the chemistry data are not yet available, we found that mutation frequencies were not significantly elevated at the selected reference sites and that there were no apparent positive interferences in the application of the test to pore water exposures.

To further characterize environmental factors that may affect baseline mutation frequencies induced following field exposures, we have recently evaluated the effects of ultraviolet light (UV-B) on the nematode. Although, the effects of UV have been evaluated before (Coohill, 1988; Hartmann, 1984 a,b), these studies have not utilized wavelengths that are typical of solar UV exposures, nor have they utilized chronic exposures typical of field situations. We conducted chronic exposure experiments using wavelengths representative of solar light and assessed the number of F1 progeny following one generation of exposure. We observed significant decreases in fecundity at doses that were below those that would be incurred following exposure to solar light at temperate latitudes. These data indicate that it may be wise to shield nematodes from incident UV during exposures. Consequently, nematode tests may be conducted

onsite immediately following sampling, and exposures may be incubated in any receptacle that omits light and provides crude temperature control (15 to 25^{0}C). Poor characterization of baseline mutation frequencies is a problem that has haunted other attempts to introduce new environmental assays in a rigorous manner. Consequently, we have emphasized detailed initial characterization.

To evaluate the sensitivity of the nematode assay to selected environmental mutagens, we must first determine the inherent activation and detoxication potential of the nematode as well as the potential for introducing exogenous metabolic activation systems into the assay design. These studies are currently underway, and it is anticipated that further evaluations of the response of the nematode test to standard mutagens and environmental chemicals will follow.

Comparing the Sensitivity of Sea Urchin Fertilization, Development, and Anaphase Aberration Assays to Three Genotoxic Compounds

One of the goals of our program is to determine whether genotoxicity tests are more sensitive than conventional ecotoxicological assays. Toward this end, we are in the process of comparing the sensitivity of sea urchin fertilization and development assays to the sensitivity of a sea urchin embryo anaphase aberration assay. These experiments are significant for two additional reasons. First, the anaphase assay is widely regarded as a convenient technique; yet, it has not been validated using standard chemicals in solution. Secondly, the existing database on sublethal effects of most genotoxic substances is remarkably poor. If it is found that conventional tests are reasonably sensitive, we may have more confidence in predictions made from the existing data.

The experiments are being conducted in two phases. We first screened the sensitivity of the fertilization assay to five genotoxic chemicals. Subsequently, we selected three substances for dose response studies using the development and anaphase aberration assays. The substances that have been tested using the fertilization, development, and anaphase aberration assays are benzidine, pentachlorophenol, and phenol. For both benzidine and phenol, the sea urchin fertilization assay was as sensitive as any other species tested to date. All of the exposures have been conducted using radiolabelled compounds, and exposure concentrations have been monitored using liquid scintillation counting.

Evaluating Genotoxicity at Field Sites in San Francisco Bay

The laboratory research described above has created the foundation for field validation studies to be conducted in San Francisco Bay. In the spring and summer of 1992, we will compare the sensitivity of the nematode mutagenesis test, the sea urchin anaphase aberration test, and the Ames test following exposure to pore waters collected from contaminated sediments. In addition, we will obtain data on sediment toxicity using

conventional short-term toxicity tests; these tests measure only lethality as an endpoint. Availability of this data using the same sites will allow us to compare the sensitivity of the genotoxicity tests to the sensitivity of the conventional short-term tests. For all of the sites, extensive chemical characterization data will also be available.

Predicting the Potential Ecological Significance of Short-Term Genotoxic Responses

The adequacy of short-term genotoxic responses to predict detrimental effects at the organismal and population levels is often debated; but rarely, can experimental data be linked to conceptual or predictive models. Anderson and Harrison (1991), synthesized data they had previously obtained comparing chromosomal aberrations, sister chromatid exchanges, dominant lethal mutations, and reproductive success of the polychaete worm *Neanthes arenaceodentata* in response to exposure to ionizing radiation. They found that the levels at which short-term responses, such as chromosomal aberrations and sister chromatid exchanges, were induced were predictive of the levels at which alterations in reproductive success also occurred. In addition, it was demonstrated that the effects of chronic exposures may be cumulative. Significantly, they also evaluated the relative significance of various processes that may mitigate our ability to extrapolate effects occuring at the chromsomal level to effects on populations. These processes included such factors as mitotic delay, interphase cell death, varied sensitivity of gametogenic stages and synchrony of gametogenesis. To our knowledge, such factors have not been characterized in any other genotoxicity research using aquatic organisms, and this is the first such conceptual model developed to identify significant processes. Moreover, there has only been one other study which has correlated response levels of cytogenetic effect to alterations in reproductive success of an aquatic species (Liguori and Landolt, 1985).

To date, efforts in ecotoxicology to evaluate the potential population-level impacts of toxic substances have not considered the multigeneration effects of genotoxic substances. Clearly, genotoxic contaminants elicit effects that are transmitted to subsequent generations, and this factor requires further consideration. We have developed a simple model to provide a preliminary evaluation of the significance of inherited lethal mutations on population size of a selected species. We have used data obtained in the study described above on the polychaete worm, *Neanthes arenaceodentata*, exposed to ^{60}Co for 1.5 generations. This work has been conducted by Dr. Anderson, Dr. Florence Harrison (Lawrence Livermore National Laboratory) and Professor John Harte (Universty of California, Berkeley).

The data analyses for the 300 broods studied are complex; however, the projected F2 population size for the controls is 197; whereas the projected F2 population size for a dose of 0.17mGy/h is 8. In contrast, there was no significant difference in the number of juvenile F1 worms in the irradiated groups as compared to the control. These findings demonstrate that through collection of appropriate life history data in ecotoxicological studies, population-level impacts of multigeneration exposures to genotoxic contaminants can be estimated and that multiple generation exposures may be necessary to adequately

predict the effects of genotoxic substances. Ecological studies in 1992 and 1993 will include mesocosm testing and multigeneration exposure experiments using additional species.

Management Recommendations: Development of a Strategy for Incorporating Genetic Toxicology into Ecological Risk Assessment

Over the next few years, we plan to provide managers with field-validated biomonitoring approaches that can be used for widespread application. In addition, we plan to perform studies to determine the potential ecological significance of these responses, and to evaluate the sensitivity of the new tests in relationship to the sensitivity of existing toxicity tests that do not assess sublethal effects. With this information in hand, we will be able to help answer the question "How can genetic toxicology be incorporated into ecological risk assessment?"

Ultimately, an integrated strategy is needed to ensure that progress in this area addresses the highest priority needs of hazardous waste management. For example, it is essential that any strategy include the development of a minimum of three tests for all of the varied media such as soil, sediment, sludge and water. Tests should be developed that are adequate for field testing, and a suite of longer term tests is needed for selected applications. These and other concerns must be identified and prioritized in the years to come. Additionally, as research advancements, such as the development of new biomarker techniques and the characterization of mechanisms of mutation, become available, these technologies must also be incorporated into biomonitoring studies of sentinel organisms at waste sites.

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