First Year Progress Report for EMSP-50

Determining Significant Endpoints for Ecological Risk Analyses

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INTRODUCTION

This report summarizes our first year's progress of research funded under the Department of Energy's Environmental Management Science Program. The research was initiated to better determine ecological risks from toxic and radioactive contaminants. More precisely, the research is designed to determine the relevancy of sublethal cellular damage to the performance of individuals and to identify characteristics of non-human populations exposed to chronic, low-level radiation, as is typically found on many DOE sites. We propose to establish a protocol to assess risks to non-human species at higher levels of biological organization by relating molecular damage to more relevant responses that reflect population "health". We think that we can achieve this by coupling changes in metabolic rates and energy allocation patterns to meaningful population response variables, and by using novel biological dosimeters in controlled, manipulative dose/effects experiments. We believe that a scientifically defensible endpoint for measuring ecological risks can only be determined once we understand the extent to which molecular damage from contaminant exposure is detrimental at the individual and population levels of biological organization.

FIRST YEAR'S PROGRESS

Development of Molecular Probe

We are using a new method to measure stable chromosomal aberrations known as reciprocal translocations. Stable aberrations, unlike others, remain detectable long after the initial exposure. The technique has been applied to Japanese atomic-bomb survivors and has shown stable aberrations in blood cells of individuals who received radiation exposures over 50 years ago (Lucus, et al. 1992). The methodology was a spin-off of the human genome project of the Department of Energy and was made possible when complete 'libraries' of DNA sequences unique to each individual human chromosome became available (Pinkel et al. 1988). In principle, the same approach could be used to study damage in the chromosomes of any organism. Until recently, however, technique development and application of the approach for ecological risk analyses would have

required a monumental effort for each species studied. It is now possible to achieve the same goal, with much less effort in isolating the necessary probes, by using chromosome microdissection techniques followed by polymerase chain reaction (PCR) amplification. Using these techniques we should be able to develop a biological dosimeter sensitive to low-level chronic exposures, and thus estimate doses to individuals with unknown exposure histories. We can then determine if there is a measurable relationship between the accumulation of sublethal chromosome damage and ecologically relevant parameters of individuals (such as metabolic rates, growth rates, age-specific survivorship, reproductive output, age at maturity and longevity).

During this first year, our collaborators at Colorado State University (CSU) began by establishing a turtle fibroblast cell line in culture. This was accomplished by using eggs from a gravid female slider turtle ***(Trachemys scripta)*** from the Savannah River Site. The turtle was induced to lay eggs by injection of a smooth muscle contraction hormone (oxytocin). Egg shells were decontaminated of adhering bacteria and embryos were used to start the cell lines of turtle fibroblasts.

Next, CSU developed a probe which selectively binds to one of the larger chromosomes. This involved perfecting the microdissection and PCR protocol for the turtle chromosomes, and preparing turtle repetitive DNA for a blocking reagent. Results, using fluorescent in situ hybridization as illustrated in Figure 1, demonstrate that the probe binds only to chromosome number 1. Our colleague, Dr. Muhlman-Diaz, has also developed a probe to bind to four of the smaller chromosomes and these probes are currently being perfected. Simultaneous use of multiple probes could significantly increase the response sensitivity in future irradiation and toxicity experiments.

With the probe for chromosome 1 developed, we began farming cells for use in a series of cellular experiments. The idea is to use experiments at the cellular level to identify promising research directions, then proceed to studies using live turtles. In this way, we can limit the use of live organisms to those studies which have the highest probability of yielding useful results.

We have completed our first cellular experiment-an acute irradiation of turtle fibroblasts. Replicate flasks of contact-inhibited, non-cycling cells were y-irradiated with 0 (control), 4, 6, 8, and 10 Gy at a dose rate of 1.95 Gy / minute. Following a delay to allow for repair, each flask was subcultured into five separate, additional cultures. Beginning at 24 hours, colcemide was added to one culture from each dose and the cells were fixed six hours later. This was repeated at six hour intervals. Cells were then stained on slides and scored for determination of mitotic index. The procedure allowed us to determine the time period when cells reached the first post-irradiation mitosis for each dose.

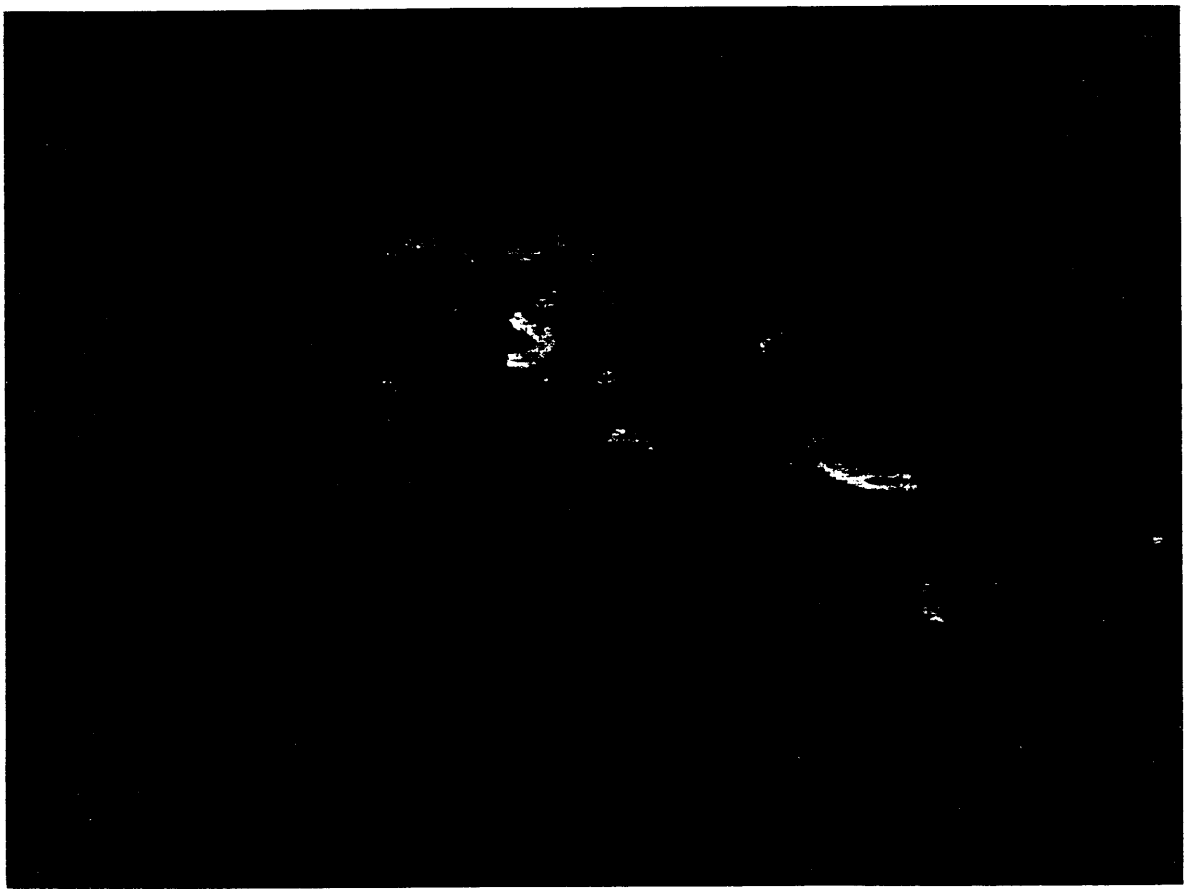

Figure 1

In situ **hybridization of biotinylated turtle whole chromosome 1 specific probe to turtle metaphase cells. Detection (yellow) with Fluorescein labelled avidin. Counterstaining was with propidium iodide (red)**

We are currently scoring all cells for total aberrations and then will use the fluorescent probe for chromosome 1 to determine the frequency of reciprocal translocations. We have already identified several reciprocal translocations in irradiated turtle cells using the probe (Figure 2). To our knowledge, it is the first time this technique has been successfully used on a non-mammalian species. One cell culture from each flask was retained to study the long-term stability of reciprocal translocations. These cells will be used to examine a time series of translocations over several cell generations.

From this experiment we will obtain: (1) the efficacy of the probe in detecting translocations, (2) the mitotic delay for a range of doses, (3) two dose-response curves for acute irradiation (one for total aberrations and one for reciprocal translocations), and (4) the stability of induced reciprocal translocations over time.

The next series of cellular experiments will investigate: (1) the effectiveness in inducing reciprocal translocations of chronic vs. acute irradiation, (2) interactions between exposure to radiation and exposure to mutagenic metals such as chromium and mercury found at the SRS, and (3) genomic instability in ***T. scripta.*** The theory of genomic instability, for which there is some evidence in other species, is that the progeny of previously irradiated cells may be more susceptible to further irradiation than the progeny of unirradiated cells. This would indicate damage to a cellular control mechanism, rather than direct radiation damage.

The use of live turtles in future experiments requires the development of a nonlethal method of obtaining blood samples from individual animals. Repeated blood samples from irradiated individuals are needed to investigate the stability of reciprocal translocations. Knowledge of aberration stability is critical to assessing any long-term, cumulative effects in chronically exposed individuals. We have worked with personnel from the CSU Veterinary Teaching Hospital (VTH) to develop a repeatable, nonlethal, sterile blood sampling technique. In November, veterinary surgeons will attempt to install a port in the plastron from which we can repeatedly draw blood samples from the heart. This technique has been successfully used at the VTH on terrestrial species of turtles without lasting ill-effects.

Field Experiments on the Savannah River Site

We conducted an experiment to compare the effects of environment on mosquitofish (Gambusid and snails (Camneloma) reared in a radiation site (H-Area basins), metal contaminant site (coal ash basins) and a control pond (Fire Pond). The study was conducted from May to October, 1997. Animals were kept within enclosures at each site for two months, then removed to the laboratory and measured for standard metabolic rate

Figure 2

In situ hybridization of bitionylated turtle whole chromosome 1 specific probe to irradiated turtle metaphase cells.
Reciprocal translocation (white arrows) detected (yellow) with Fluorescein labelled avidin. Counterstaining was with propidium iodide (red).

(SMR). One-third of the organisms from each site were to be returned to their site of initial exposure, and one-third would be transferred to each other site for the second half of the study (two months). The design resulted in nine treatment for each species (TABLE 1); pair-wise comparisons of treatments allowed for tests of impacts of the stress environments during different life-stages.

TABLE 1. Design used in our first field experiment to test if stress from exposure to contaminants increases metabolic rates.

Treatment	*Exposure For 1st Two Months*	*Exposure For 2nd Two Months*
1	radiation	radiation
2	radiation	trace elements
3	radiation	unpolluted
4	trace elements	trace elements
5	trace elements	radiation
6	trace elements	unpolluted
7	unpolluted	unpolluted
8	unpolluted	radiation
9	unpolluted	trace elements

The experiment began when we transplanted 12 snails each into 12 20-L mesh and plastic containers at each site, and 15 mosquitofish each into 12 70-L mesh and plastic containers at each site (total = 36 cages / species). Two months later, in mid-July, we removed surviving animals from all cages. Standard metabolic rates were estimated for survivors after which they were returned to field cages for the second portion of the study. Estimates of external dose rates to the animals at each location were derived from readings of thermoluminescent dosimeters (TLD) placed on the sediments and floating on the water surface (TABLE 2).

Results--Surprisingly, all animals transplanted to the metal-polluted site (coal ash basins) died during the first half of the study, whereas survival in the radiation site and reference site was high (means ± 1 SE: snails: 90.1 ± 3.3% [H-Area], 97.2 ± 1.6 % [Fire Pond]; mosquitofish: 71.5 ± 4.7% [H-Area], 72.1 ± 3.7% [Fire Pond]). Standard metabolic rates of snails and mosquitofish did not differ between sites after the first half of the study. Because of total mortality in the metal contaminated site during the first half of the experiment, we used only two sites for the second portion, H-Area (radiation area) and Fire Pond (reference site). Unfortunately, prior to the scheduled end of the study in

October, 1997, a prolonged drought resulted in drying of the H-Area basins, and subsequent loss of experimental animals in that site.

TABLE 2. Net dose rates (mGy / day) from external irradiation estimated from thermoluminescent dosimeters placed on the sediment surface and floating on top of the water.

Treatment	*Dose Rate at Sediment Surface*	*Dose Rate at Surface of Water*
H-Area Basin (Radiation)	2.12 ± 0.51 (n = 24)	1.31 ± 0.07 (n = 24)
Coal Ash Basin (Metals)	0.01 ± 0.002 (n = 3)	0 ± 0 (n = 6) *
Fire Pond (Control)	0 ± 0 (n = 6)*	0 ± 0 (n = 6) *

* Lower limit of detection = 0.0005 mGy / day

Because of the complete mortality of fish and snails in the coal ash site during the first half of the study, we transplanted a second group of mosquitofish to the metal-contaminated basins and Fire Pond (reference site) for one month (July - August, 1997), to determine if shorter-term exposure could provide insights into why the mortality occurred. After one month at the same densities as the initial studies, we removed survivors from each cage for measurement of SMR. After only one month of exposure, survival of fish in the metal-polluted site was very low (mean = 21%) compared to Fire Pond (75%). The low number of survivors from the metal-polluted site precluded proper measurement and analysis of metabolic rates.

Summary-- It appears that, from a relative-toxicity standpoint, conditions in the metal contaminated site are more severe than in either the radiation or reference sites. However, we were not able to quantify long-term effects on survival or sublethal responses due to early drying of the H-Area basins and subsequent loss of animals. Samples of fish and snails from H-Area and Fire Pond were sacrificed following the first half of the study, and are currently being analyzed for radionuclide concentrations.

Design and Construction of Mesocosms and Irradiation Facility

Part of our research will involve irradiating populations of fish, tadpoles, salamanders and turtles (living in mesocosms) to a range of chronic, low-level exposures using sealed ^{137}Cs sources, alone and in combination with varied concentrations of heavy metals.

Animals will be reared in mesocosms (outdoor, above-ground tanks in which artificial pond communities are established) that allow a more controlled environment than large-scale field tests. Perhaps the greatest advantage of using mesocosms is the ability to replicate treatments such that powerful statistical methods such as ANOVA can be used (Rowe and Dunson, 1994). Mesocosm experiments using a variety of organisms will allow the determination of: 1) chromosome damage at various radiation exposure and metal contaminant concentrations, 2) the relationship between cellular damage and metabolic rate, 3) the effects of treatment levels on an individual's energy allocation pattern, and 4) effects on growth and survival.

Considerable effort was spent this first year designing a mesocosm suitable for a variety of species, as we anticipate using turtles, fish and amphibians as model organisms. Because each individual mesocosm will have a sealed ^{137}Cs source suspended above it, additional design considerations were needed to homogenize the dose distribution within the mesocosms, and to minimize the dose a human would receive working in the field of 50 mesocosms. A health physicist, Dr. John Campbell, was hired to calculate the mesocosm shape such that variation in dose within the tank would be minimal, and yet animal husbandry needs met. The result was a parabolic-shaped disk, 244 cm in diameter containing 30 cm of water and a ^{137}Cs point source suspended 61.4 cm above the water surface (Figure 3). The distribution of exposures within the tank is shown in TABLE 3 as a function of water depth and distance perpendicular from the center line of the source. Because dose rate is greatest 0 to 10 cm below and 0 to 40 cm perpendicular from the source, a second, inner container was designed to contain a subgroup of animals within that area (Figure 3). By isolating an inner area, the overall spatial variation in exposure is substantially reduced, and the possibility to run two simultaneous experiments within each tank becomes possible. One experiment uses the outer portions of a tank and a second, at a higher mean dose rate, is conducted within the inner container. This design reduces variation within a treatment and increases the flexibility of our experiments. A subcontractor is currently constructing the 50 mesocosms. We expect delivery in December 1997.

The array of 50 mesocosms (Figure 4) with their associated ^{137}Cs sources produces a radiation field whose magnitude must be considered for worker safety. Two components of the radiation dose were considered: a direct component produced by radiation penetrating the source shielding and an indirect component resulting from scatter of the source beam from the water contained within the mesocosms. Dose to a worker standing in the middle of the array of mesocosms (Figure 4) and receiving radiation from the adjacent mesocosm as well as five surrounding mesocosms was calculated by Dr. Campbell. Mesocosms were

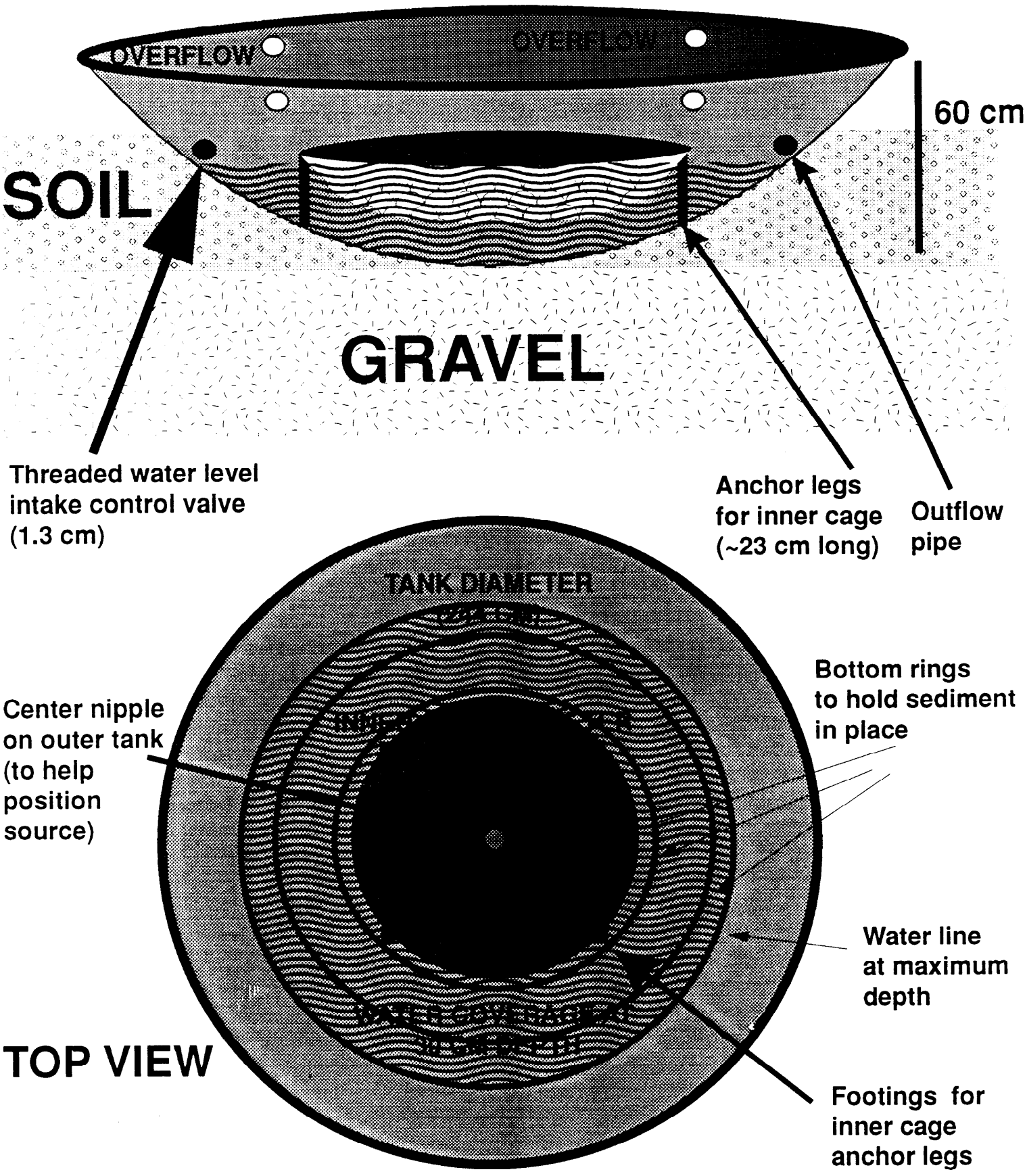

Figure 3. Design of experimental tanks for radiation and metals studies.

TABLE 3. Distributions of exposures (mR/hr/Ci) within a mesocosm tank as a function of water depth and distance perpendicular from the center line of the source.

depth (cm)	perpendicular distance from axis (cm)										
	0	10	20	30	40	50	60	70	80	90	100
1	953.9	930.8	867.9	780.2	684.1	591.0	507.3	435.0	374.0	323.0	280.6
5	948.9	928.3	871.5	790.7	699.5	608.6	524.4	449.9	385.7	331.0	
10	791.6	774.9	728.3	661.0	583.4	504.5	430.2	363.6	305.8		
15	602.9	590.2	554.5	502.2	441.3	378.7	319.3	265.8			
20	437.3	428.0	401.9	363.4	318.1	271.2	226.4				
25	307.9	301.4	282.8	255.4	222.9						
30	212.6										

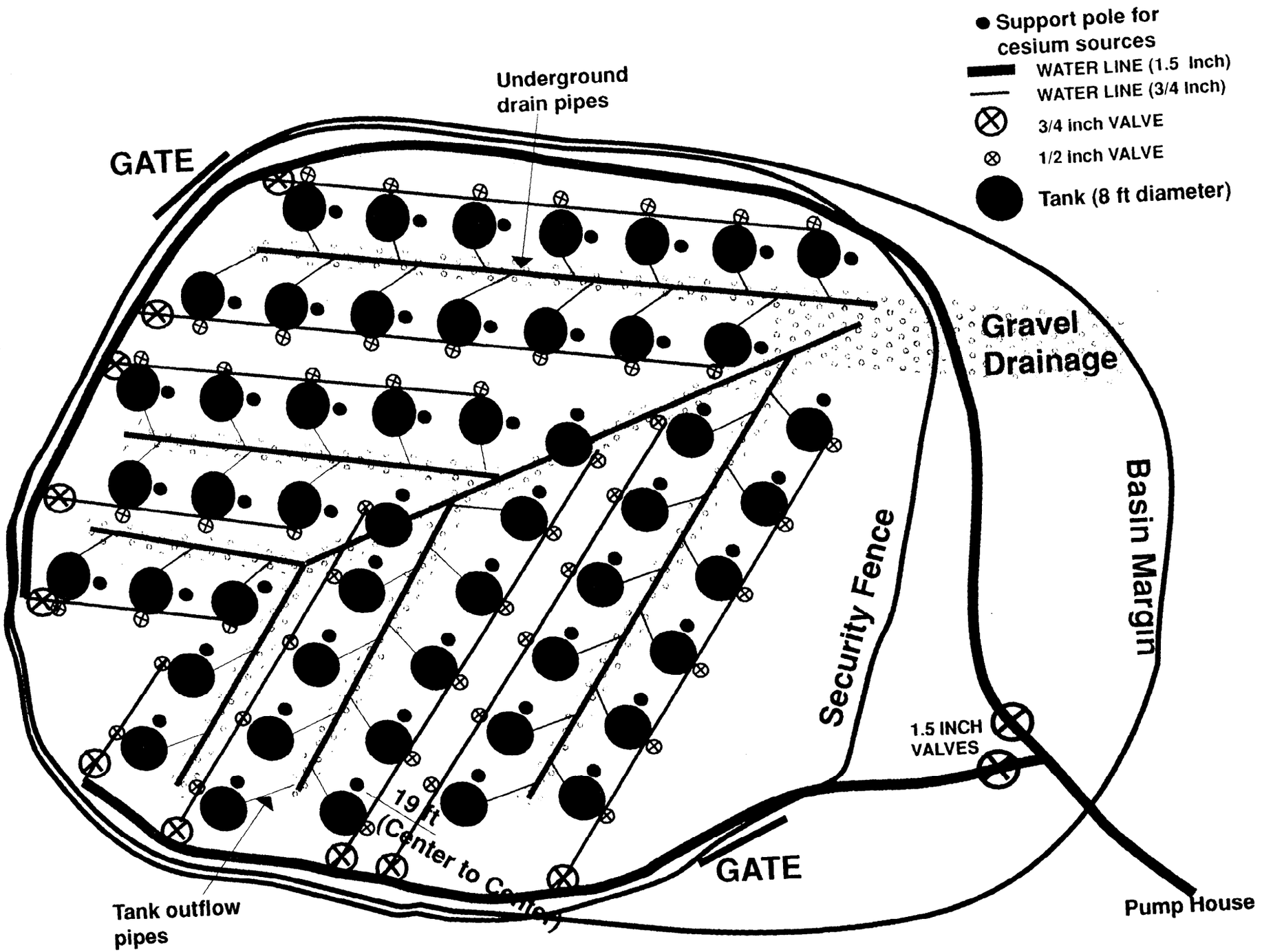

Figure 4. Overview of proposed tank farm layout and drainage plan.

assumed to be 244 cm in diameter and spaced 183 cm from one another. Assuming a 37 GBq (1 Ci) source suspended over each mesocosm, results indicate that a worker would receive 0.053 mGy / h. Dose contributions from mesocosms other that the adjacent one were minimal. Minimization of dose can be achieved by placing the higher activity sources as far as possible from each other in the array and by maximizing the distance between mesocosms. These calculations were obtained from a model designed by Dr. Campbell (Campbell and Jacobs, 1992). His data will be checked by measuring the dose distributions from the actual radiation field produced by a single ^{137}Cs source in the geometry described by the model prior to our purchasing all 50 sources for the project. The verification test is planned for November 1997.

A location on the Savannah River Site has been chosen for the array of mesocosms. Land has been cleared of trees, graded, and storm drains installed. The site was chosen due to its proximity to the PAR Pond Radioecology Laboratory, its isolation from the general public, and the availability of water and electrical power.

Acquisition of Additional Funds

Cost of the sealed ^{137}Cs sources increased because additional shielding was required for worker safer. Costs of the mesocosms was also greater than anticipated because we had to design special mesocosms to reduce the spatial heterogeneity in dose. Our initial allocation of funds from the DOE EMSP award for the sources and mesocosms would have resulted in purchasing such a small number of units that our experimental design would have been compromised. To compensate for the additional costs, we were able to solicit an additional $35,000 from the Savannah River Ecology Laboratory for purchasing the mesocosms, and $5,000 for site preparation of the irradiation facility.

CONCLUSIONS

We are quite excited about our development of the first chromosome-specific probe for a non-mammalian species. The probe, when fluorescently labeled, ‘paints’ that chromosome in any cell of that species treated with the probe. The labeled DNA is then hybridized back to cells sampled from individuals of the same species that have been exposed to contaminants. Reciprocal translocations that have occurred over the life of the organism can then be quantified by observing the fluorescent marker on translocated chromosomes (Figure 2). Thus, a biological dosimeter that measures cumulative damage in a long-lived vertebrate, the yellow bellied slider ***(Trachemys scripta), is*** now possible. Our development of this tool is fundamental to our determining what constitutes a ‘significant risk’ in ecological risk analyses. When the relationship between dose and

response (i.e. metabolic rate) is quantified, we will be able to examine effects of contaminants across levels of biological organization, and determine the significance of certain chromosomal and sublethal endpoints to higher level parameters, such as energy allocation, age-specific survivorship, reproductive output, age at maturity and longevity.

Although our first field experiment on the SRS was disappointing because of total mortality in the coal ash treatment and loss of organisms in the radiation basins due to drought., we acquired valuable dosimetry data from the radiation basins, and the lessons learned are now being applied in the design of our next experiment.

The design of the mesocosms and irradiation facility has been completed. Construction of the mesocosms is nearing completion and we will soon test the spatial distribution of dose with our pilot, sealed ^{137}Cs source. When the irradiation facility is completed we will be well suited to use the statistical power inherent in replicated mesocosms to address the response of non-human organisms to low levels of radiation exposure and metal contaminants.

Finally, we have been judicious in our spending of funds and have even used the EMSP grant to leverage an additional $40,000 from the Savannah River Ecology Laboratory for items that were more expensive that originally planned.

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