DETECTION OF DNA-DAMAGING COMPOUNDS

IN CONCENTRATED WASTEWATER

by

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CHAPTER I

INTRODUCTION

In the past few years it has become apparent that the majority of human cancers are a result of chemicals present in the environment(4). Research has been done to evaluate the role of air and food as the sources for these carcinogens, but relatively little has been done regarding water. Recently, however, investigators have begun to look at the potential health hazard of various compounds which may be found in water.

In 1977 Pelon and co-workers were able to detect a mutagenic response with histidine-dependent strains of Salmonella typhimurium (Ames system) to unconcentrated samples of Mississippi river water(14). Also in 1977, Loper and others tested concentrates of tap water from several U.S. cities using the Ames system. These samples represented different sources of water such as municipal wastes, industrial wastes, agricultural drainage and ground water. They were able to detect mutagenic activity in each sample(8). These along with other studies indicate that mutagens are found in various water sources(13,15,16). This is a significant finding since water is quickly becoming a valuable commodity.

The South Plains of Texas uses water derived from the Ogallala aquifer. This formation stretches from West Texas to Nebraska and is mined for the irrigation of crops. Unfortunately, this is rapidly being depleted and may no longer be of use within the next twenty years. This has led to the search for alternate water sources.

One alternative is reclaiming of wastewater. Lubbock, Texas, has been reusing its wastewater since 1925. In 1937 the city entered into a long-term irrigation reuse contract with a local farmer, Frank Gray. Effluent from Lubbock's water reclamation plant is pumped to the farm and stored in holding ponds(i.e., lagoons) until needed for irrigation. As a result of percolation from the holding ponds and irrigation, the water table beneath Gray's farm has rised to within a few feet of the surface. To bypass flooding the city has further utilized the reclaimed water to develop a series of recreational lakes. Water is collected

from the water table in wells and pumped to the lakes which are situated in a canyon adjacent to the farm.

Research has been done to determine the safety of this reused wastewater. Several aspects such as bacterial and viral contamination and chemical content have already been evaluated(7,17,19). The presence of harmful compounds is another parameter examined in the current study.

Water samples were collected at various stages of treatment and concentrated on XAD-2/XAD-7 columns. The concentrated samples were screened for DNA-damaging activity. DNA-damaging assays have been routinely used to test purified compounds, but this paper evaluates their usefulness in the field.

CHAPTER II

MATERIALS AND METHODS

Bacterial strains. All bacterial strains were provided by Dr. I.C. Felkner, Department of Biological Sciences, Texas Tech University. Five strains of Bacillus subtilis deficient in different steps of recombination(Rec-) and excision(Exc-) repair were used to detect DNA-damage. These were Exc- strain hcr-9 (11); Rec- strains recE4, recA8 (3), and mc-1 (12); Rec- Exc- strain fh2006-7 (6); and the isogenic strain 168 which has all repair mechanisms intact.

Chemicals and Media. The XAD-2 and XAD-7 resins were purchased from Polysciences, Inc., Warrington, PA. Dimethylsulfoxide(DMSO) was purchased from MCB Manufacturing, Chemist Inc., Cinncinnati, Ohio. Mitomycin C(Mit C) was purchased from Sigma Chemical Co., St. Louis, Missouri.

The culture media used was Difco brain-heart infusion(BHI). For the survival test, treated cells were diluted in Spizizen's minimal salts(SMS) solution(18), and plated on Difco nutrient agar.

DNA-damaging assay. The DNA-damaging activity of each sample was determined within five days after collection. The primary lagoon sample collected 10/6/80 was retested after three months and then eight months of storage at 5°C. Samples from the secondary lagoon and the canyon lake were tested with and without activation with S-9 mix(1). The DNA-damaging assay was performed according to the procedure of Felkner(5). Each strain was grown overnight at 37°C in BHI, then streaked radially on a nutrient agar plate to a centered disc containing 0.05 ml of the sample. Included in each assay were Mit C (0.01 mg/0.05 ml DMSO) amd DMSO as positive and negative controls respectively. Each sample was run in triplicate. After overnight incubation at 37°C, inhibition was determined measuring the distance from the edge of growth to the disc.

A quantitative test was performed on the primary lagoon sample (10/6/80) for the strain found to be most sensitive to the sample(i.e., fh2006-7) and the wild-type 168. Side arm flasks with 15 ml of BHI each were inoculated with the test strains then incubated with shaking

at 37°C until an OD_{540} of 0.3 had been obtained. The cultures were diluted with fresh BHI to an OD_{540} of 0.16, then divided into 1.0 ml aliquots in sterile polystyrene screwcap tubes. Various concentrations of the sample dissolved in 0.01 ml of DMSO were then added to each aliquot. After thirty minutes of incubation with shaking at 37°C, each culture was subsequently diluted in SMS to the estimated range and plated on nutrient agar. After incubation at 37°C for 1-2 days, viable colonies were counted. The fractional survival(N_t/N_o) was determined at each concentration by the following equation:

$$\text{Fractional survival} = \frac{\text{Number of viable colonies from treated cells}}{\text{Number of viable colonies from untreated cells.}}$$

Concentration of water samples. Columns were prepared according to the procedure of Donnelly(2). The XAD-2 and XAD-7 resins were washed by swirling and decanting three times with ten volumes each of acetone, methanol and distilled water. Glass columns(15mm i.d. by 600mm) were filled with distilled water. Approximately 15 cm^3 of each resin was added slowly to prevent formation of air pockets. The mixed-bed column was then rinsed with thirty bed volumes of distilled water before use.

Water was collected in opaque bottles from various sites(Fig. 1). Each water sample was then passed through the XAD-2/XAD-7 column by gravity flow. The excess aqueous phase was removed with dry nitrogen. Adsorbed organic compounds were eluted from the column with acetone. This mixture was reduced to a few milliliters using a flash evaporator; transferred to a sterile polystyrene test tube and taken to dryness under a stream of nitrogen. The residue was dissolved in an appropriate amount of DMSO to achieve a one to three thousandfold concentration then stored at 5°C until assayed.

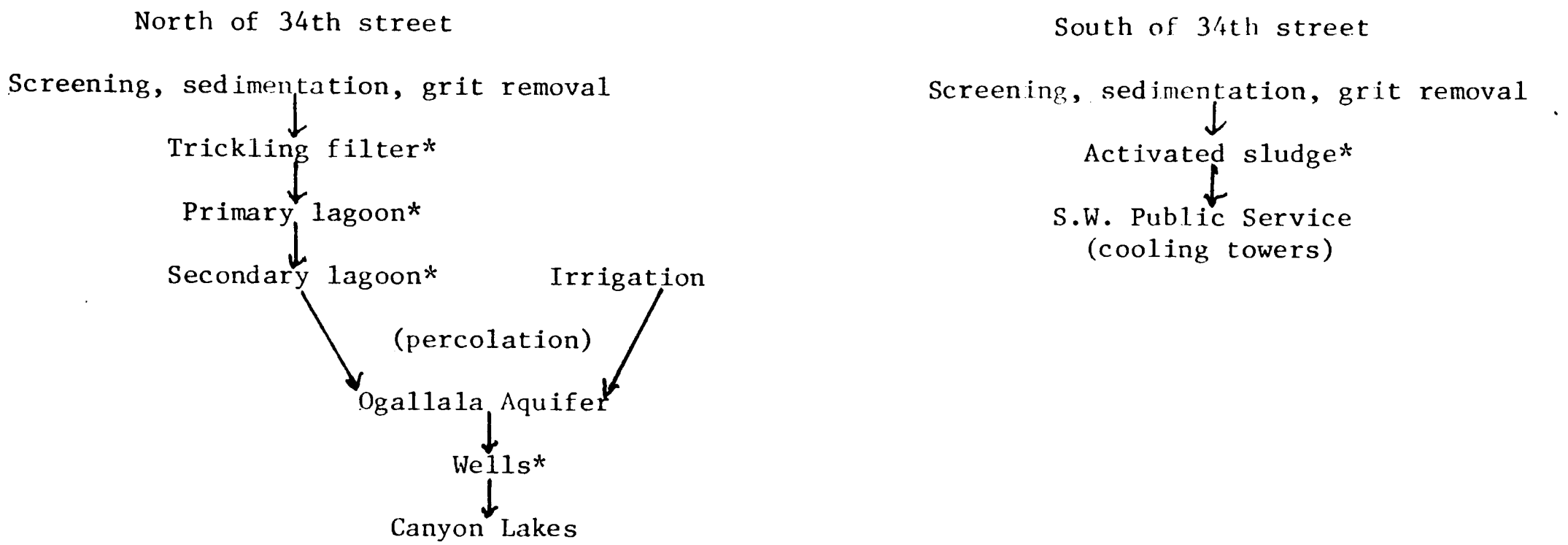


Figure 1. Schematic representation of Lubbock's water treatment process.

* Indicates locations samples were taken.

CHAPTER III

RESULTS

DNA-damaging test. The results of the DNA-damaging test are shown in Tables 1-4. Both solvent and positive controls are included for comparison.

Table 1 contains the results of samples taken from the treatment plant. All strains showed significant inhibition by the sample from the activated sludge. The wild-type strain 168, with all repair mechanisms intact, was inhibited to the same extent as the repair deficient mutants. Strains showed various responses to the trickling filter sample. Strain 168 displayed no sensitivity to the sample. The repair deficient mutants showed different degrees of inhibition, the greatest being displayed by mc-1 and fh2006-7.

Table 2 contains the results of assays from samples of the primary lagoon taken on different days. DNA-damaging activity was present in samples collected 8/8/80 and 10/6/80 although no activity was found in the sample collected 2/6/81. The sample collected on 8/8/80 has no affect on the wild-type; all other strains, both Rec- and Exc-, showed significant inhibition.

The sample taken on 10/6/80 showed the greatest degree of DNA-damaging activity on strains mc-1 and fh2006-7. All other strains, including the wild-type, displayed various degrees of sensitivity with recA8 showing only slight inhibition.

The primary lagoon sample collected 10/6/80 was retested after storage at 5°C for three months and then eight months. The results of these assays are shown in Table 3. DNA-damaging activity was present after three months although most of the strains' responses changed. Strain hcr-9 showed no inhibition to the three-month-old sample. Other strains which demonstrated a decreased sensitivity to the sample were 168 and mc-1; recA8 showed an increased sensitivity. When the sample was tested after eight months of storage, no DNA-damaging activity was detected as shown by the lack of growth inhibition in any of the six strains.

Table 4 contains the results of samples taken from locations dis-

tant to the primary lagoon(i.e., secondary lagoon, well and Canyon lake). No DNA-damaging activity was detected in these samples.

Figure 2 is the fractional survival of fh2006-7 and 168 to increasing concentrations of the sample from the primary lagoon. It shows an obvious decrease in the survival of the Rec-, Exc- strain fh2006-7 when compared to the wild-type 168.

Table 1. Results of DNA-damaging assays in samples from the water treatment plant.

Samples	Equivalent amount	Strain 168	mc-1	fh2006-7	recE4	recA8	hcr-9
		Inhibition radius[a]					
Mit C	.05 ml	++	+++	++++	+++	+++	+++
DMSO	.05 ml	-	-	-	-	-	-
Activated sludge	100 ml	++	++	++	+	++	++
Trickling filter	100 ml	-	++	++	+	±	±

[a] Inhibition values for a known mutagen is included for comparison. Each strain was streaked radially on a BHI agar plate to a disc containing .05 ml of the sample and incubated overnight. The inhibition was measured in mm from the edge of the disc to the edge of growth. The values assigned for the approximate inhibition are: -, no inhibition; ±, 1 mm; +, 2 mm; ++, 5 mm; +++, 10 mm; ++++, greater than 10 mm.

Table 2. Results of DNA-damaging assays in samples from the primary lagoon collected at various dates.

Samples	Equivalent amount	Strain 168	mc-1	fh2006-7	recE4	recA8	hcr-9
		Inhibition radius[a]					
Mit C	.05 ml	++	+++	++++	+++	+++	+++
DMSO	.05 ml	-	-	-	-	-	-
08/8/80	50 ml	-	++	+	++	+	++
10/6/80	150 ml	+	++++	++++	++	+	++
02/6/81	100 ml	-	-	-	-	-	-

[a] Inhibition values for a known mutagen is included for comparison. Each strain was streaked radially on a BHI agar plate to a disc containing .05 ml of the sample and incubated overnight. The inhibition was measured in mm from the edge of the disc to the edge of growth. The values assigned for the approximate inhibition are: -, no inhibition; ±, 1 mm; +, 2 mm; ++, 5 mm; +++, 10 mm; ++++, greater than 10 mm.

Table 3. Retest of the primary lagoon (10/6/80)

Time of assay	Strain 168	mc-1	fh2006-7	recE4	recA8	hcr-9
	Inhibition radius[a]					
Zero time	+	++++	++++	++	±	++
3 months	±	+++	++++	++	+	-
8 months	-	-	-	-	-	-

[a] Each strain was streaked radially on a BHI agar plate to a disc containing .05 ml of the sample and incubated overnight. The inhibition was measured in mm from the edge of the disc to the edge of growth. The values assigned for the approximate inhibition are: -, no inhibition; ±, 1 mm; +, 2 mm; ++, 5 mm; +++, 10 mm; ++++, greater than 10 mm.

Table 4. Results of DNA-damaging assays in samples from various locations distant to the primary lagoon.

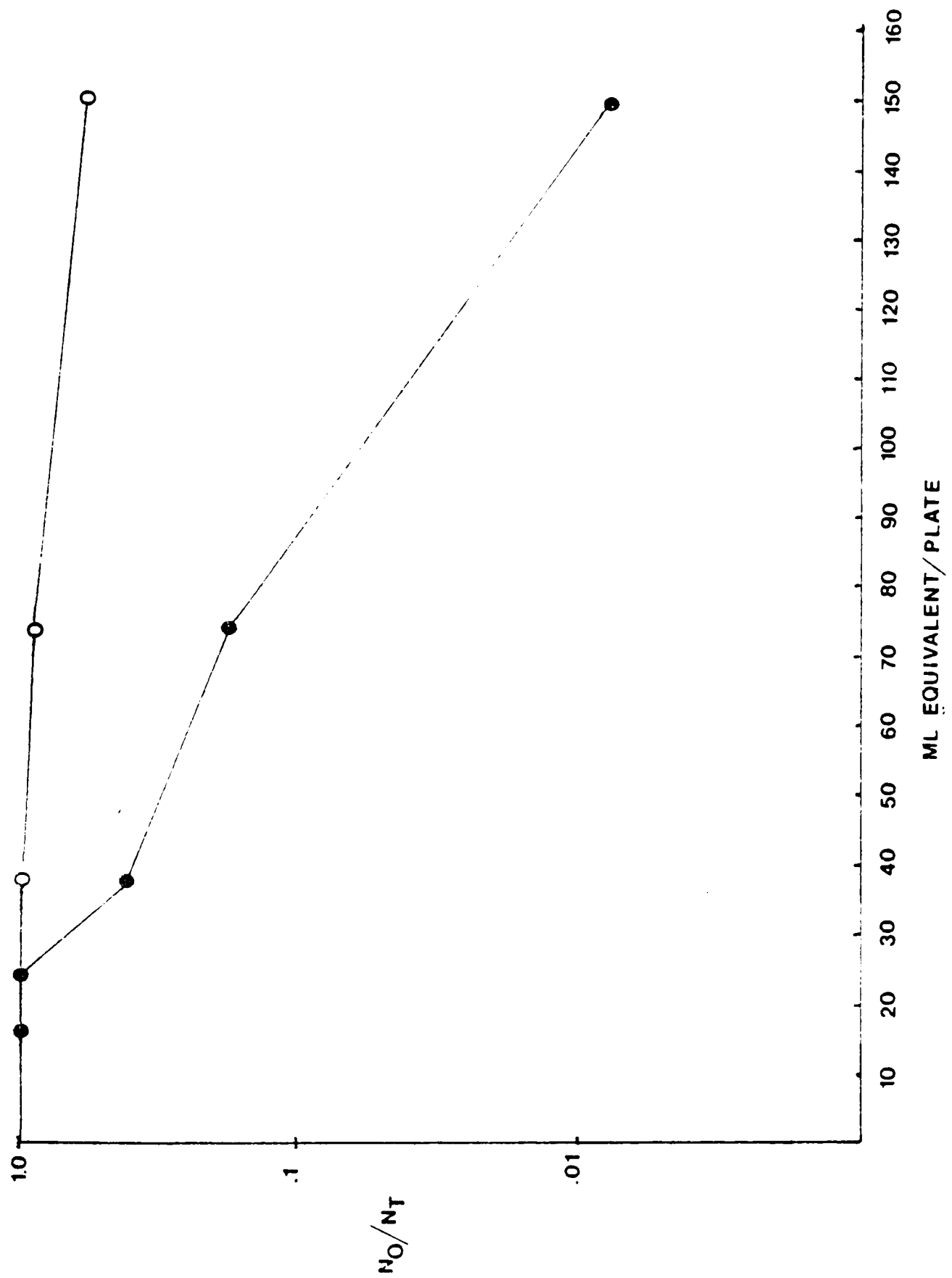
Samples	Equivalent amount	Strain 168	mc-1	fh2006-7	recE4	recA8	hcr-9
		Inhibition radius[a]					
Mit C	.05 ml	++	+++	++++	+++	+++	+++
DMSO	.05 ml	-	-	-	-	-	-
Well	50 ml	-	-	-	-	-	-
Secondary lagoon	100 ml	-	-	-	-	-	-
Secondary[b] lagoon	100 ml	-	-	-	-	-	-
Canyon Lake	50 ml	-	-	-	-	-	-
Canyon[b] Lake	50 ml	-	-	-	-	-	-

[a] Inhibition values for a known mutagen is included for comparison. Each strain was streaked radially on a BHI agar plate to a disc containing .05 ml of the sample and incubated overnight. The inhibition was measured in mm from the edge of the disc to the edge of growth. The values assigned for the approximate inhibition are; -, no inhibition; ±, 1 mm; +, 2 mm; ++, 5 mm; +++, 10 mm; ++++, greater than 10 mm.

[b] Activated with S-9 mix.

Figure 2. Fractional Survival of Strain fh2006-7 and 168 to the Primary Lagoon Sample Collected 10/6/80.

Fractional survival was determined at increasing concentration of the sample as described in the "Materials and Methods" section. Open circles(o--o) represent the fractional survival of strain fh2006-7 and closed circles(•--•) represent the fractional survival of strain 168. Mid-log cultures of each strain were divided into 1.0 ml aliquots. Various concentrations of the sample was added to each aliquot. After 30 minutes of incubation with shaking at 37°C, each culture was subsequently diluted in SMS and plated on nutrient agar. Viable colonies were counted after incubation.


1.0
.1
.01
N_O/N_T
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
ML EQUIVALENT/PLATE

CHAPTER IV
DISCUSSION

The results of the DNA-damaging test clearly indicate that there was DNA-damaging compounds present in the Lubbock wastewater at the treatment level. This was demonstrated in the sample from the trickling filter. Although the wild-type 168 was unaffected, the five repair-deficient strains showed inhibition. The growth inhibition which resulted from the activated sludge sample may not be DNA-damaging activity. All strains including the wild-type were affected the same, therefore, growth inhibition may be due to some other physiological toxicity. This toxic effect would mask DNA-damaging activity and therefore make the interpreting of the results of this sample difficult. The detection of a toxic response is a necessary control because in many water treatment plants chlorination is part of the treatment; it is important to distinguish between this type of toxicity and DNA-damage.

DNA-damaging compounds were also demonstrated in the primary lagoon. This was seen quantitatively in the determination of the fractional survival of the Rec-, Exc- strain. It can be demonstrated that the decreased survival of the fh2006-7 strain compared to the wild-type 168 can be attributed to DNA-damage because the two strains are identical with the exception of their ability to repair DNA.

It is interesting to note that the chemicals were not present at all times and may represent either a seasonal variable or a one time "industrial spill" of compounds with limited half-lives. The last sample taken from the primary lagoon in February of '81 showed no DNA-damaging activity although the water sample was concentrated two thousandfold. Another interesting fact is that the compounds were not stable as shown in the retesting of the primary lagoon sample. After eight months of storage at 5°C all DNA-damaging activity was lost.

Samples tested from other locations of the Gray farm, two of which were also activated, contained no detectable DNA-damaging activity. Therefore, from this standpoint, the water is safe for recreational use. The lack of DNA-damaging compounds may be attributed to one or

more events. First, percolation through the soil adsorbed organic compounds thereby removing DNA-damaging activity. Secondly, water may have been retained in the primary lagoon long enough so that by the time water reached other sites, compounds had decomposed. Thirdly, exposure to sun and/or air may have led to the inactivation of compounds. Other possibilities are degradation by microbes of the compounds or dilution of the chemicals to levels which were no longer detectable.

Different repair deficient mutants were used to give a range of sensitivity in order to increase the change of detecting DNA-damage. The DNA-damaging compounds present in the activated sludge and primary lagoon required both excision and recombination mechanisms for repair. This was demonstrated by the fact that the Exc- strain, hcr-9 was inhibited as were the Rec- strains, mc-1, recA8 and recE4. The increased response to the active samples of the Exc-, Rec- strain fh2006-7 over the other strains further illustrated this point.

The source for the DNA-damaging compounds has not been determined. One possibility is accumulation of pesticides used on the farm. This is unlikely because activity was not detected in samples from other locations on the farm. The more feasible possibility is contamination from one of the many industries using the water treatment plant where DNA-damaging activity was found in a sample taken from the trickling filter. These industries include a cotton oil mill, a manufacturer of electronic equipment, dairies, and food processing plants.

The presence of DNA-damaging activity is significant since similar damage to DNA is thought to be the primary cause of cancer(9), and genetic birth defects among other adverse affects. The actual risk due to the activity found in these water samples is hard to assess. The chemicals were present in trace amounts because water samples were concentrated one to three thousand times in the assay. A previous study indicates that some environmental carcinogens accumulate in body fat(10). Continual exposure to even trace amounts of these may initiate DNA-damage and therefore become a potential health-hazard.

The system in the current study provides excellent means to determine if wastewater is free from DNA-damaging compounds. The entire procedure of water concentration and running the DNA-damaging assay can be

completed in three days. Therefore this method can be used to monitor wastewater at regular intervals. If DNA-damaging activity is detected again, water from locations other than the primary lagoon could be used for irrigation; effluent from the suspect companies could be checked by this system and the potential health risk evaluated.

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