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Genotoxicity of Radiographic Photofilm Wastewater: Influence of the Treatment with a Metal Exchange Unit

H. E. Muzio, A. Magdaleno, J. Moretton

Hygiene Department, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Junin 956, Piso 4°, CP 1113AAC Buenos Aires, Argentina

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X ray imaging, or radiography, is used to assist diagnosis of a wide variety of health care professions such as oncology, dentistry, orthopedics and surgery. The vast majority of medical x-rays are recorded on silver based radiographic films, similar to white and black photographic films. Manufacturing of radiographic films accounts for 10% of the world's consumption of commercial grade silver (Nordberg and Gerhardsson 1988). Population growth and aging are expected to drive continued growth of radiographic film use despite the introduction of digital systems.

Photofilm processing generates a complex mixture of organic and inorganic compounds in the wastewaters, in the form of used film fixer solutions that contain significant amounts of silver, often exceeding 3.000 mg/L. Hydroquinone, present in virtually all black and white photographic developers, decomposes irreversibly with the time to form oxidation products. Hydroquinone has demonstrated genotoxic activity (Koike et al. 1988; Nakamura et al. 1980; Whysner et al. 2004). Sulphite, thiosulphate and trace quantities of heavy metals are also found in the effluents (Pethkar and Paknikar 2003). Certain types of solutions used in photographic processing are highly alkaline or very acidic.

Silver can be recovered from processing solutions by a variety of methods. Metal exchange is the cheapest form of recovery and is suited to the small volume users. The method involves exchange of iron, in the form of steel wool, with silver releasing the iron into the wastewater.

A mutagenic hazard can be manifested as a heritable change resulting from germ-line mutations and or somatic mutations leading to cancer or other chronic degenerative processes such as aging and coronary hearth diseases. Although there are species differences in metabolism, DNA repair, and other physiological processes affecting chemical mutagenesis, the universality of DNA and genetic code provides rationale for using various non-human test systems to predict the intrinsic mutagenicity of test chemicals. Even when the genotoxicity of compounds like silver or hydroquinone were widely studied, no data of the mutagenicity of the complex mixture generated in the photofilm processing wastewater were reported.

Correspondence to: J. Moretton

The aim of the present work was to analyze the genotoxic pollution load of the radiography wastetewaters before and after treatment by a metal exchange system for silver recovery. A tree-test battery approach was used. Ames *Salmonella* histidine reversion system, *Saccharomyces cerevisiae* D7 tests and *Bacillus subtilis* rec assay, were selected to determine the genotoxic activity in wastewater samples.

The Ames test is based on detecting reverse mutations in two histidine-auxothrophic mutants of the bacterium *Salmonella typhimurium* rendering them histidine prototrophs. One mutant strain (TA 100) allows the detection of base substitution mutation, the other one, (TA98), of frame shift mutation. This test is a reference in chemical mutagenicity testing and was extensively validated (Kubo et al. 2002)

Mitotic gene conversion is a rearrangement of genes on one chromosome. It is considered to be a final genetic event consequence of an active repair process at the DNA segment in which the damage has been induced chemically. This endpoint in the *Saccharomyces cerevisiae* D7 test is analogous to the phenomena that occur in the inhibition of growth in some DNA reparation deficient bacteria strains (Zimmermann 1984).

Bacillus subtilis rec assay measure DNA damage that is expressed as growth inhibition. The test system employs paired, isogenic cells, one has the normal DNA repair capability and one is deficient in specific enzymes responsible for the repair of damaged DNA. Preferential growth inhibition of the repair deficient strain implies that the compound being tested is reacting with the cellular DNA to produce a repairable DNA lesion and therefore may be mutagenic (Mazza 1982; Takigami et al. 2002).

MATERIALS AND METHODS

The source of test samples was wastewater obtained before (raw samples), and after treatment (treated samples) with a metal exchange compact unit for radiographic waste processing, produced by CTyT Company in Argentina. This unit is in operation in 3 Buenos Aires city area hospitals. The process is based on the exchange of iron, in the form of steel wool, with silver. This method was very successful at keeping silver levels below 5 mg/L. A simultaneous reduction in the organic matter was also achieved. BOD_5 mean values for 5 samples were 152 mg/L for raw samples, and 6 mg/L for treated samples. The values obtained in the COD test were 71,000 mg/L and 50 mg/L for raw and treated wastewaters respectively.

Each sample was submitted to biological assays either after sterilization by filtration through a 0.22 µm pore-size cellulose nitrate filter (Millipore), or as an extract obtained as follows: 500 mL of water were filtered (Whatman microfibre paper) and then passed through a column (10 cm high x 1 cm diameter) of XAD-2 resins with a flow rate of 10 mL/min. Ethyl ether was used for the resins elution.

Then the ether extracts were collected in beakers and brought to dryness by rotary evaporation at 37° C. Finally, the dried extracts were dissolved in 5 mL of dimethyl sulfoxide (DMSO), and assayed for genotoxicity (Moretton et al. 1990; Moretton et al. 1991).The final volumetric concentration factor was 100.

All the samples were assayed, at least, over 3 log concentrations range up to the limit imposed by toxicity of the sample for the tester strains.

Dr. Bruce Ames (University of California, Berkeley, CA. USA) kindly provided *Salmonella typhimurium* TA98 and TA100 strains. The assay was carried out using the plate incorporation technique (Kubo et al. 2002). The whole samples were tested with and without rat liver microsomes (S9). Phenobarbital and βnaphtoflavone-induced rat liver S9 fractions for microsomal activation were prepared and used according Maron and Ames (1983). Criterions of positive results in this bioassay were those defined by Mortelmans and Zeiger (2000): (a) two fold or greater increase in the number of revertants exposed to the test material over spontaneous reversion rates and (b) a reproducible dose-response relationship.

The diploid D7 strain in *Saccharomyces cerevisiae (MATa/MATα, ade2-40/ade2-119, trp5-12/trp5-2, ilv1-92/ilv1-92)* was obtained from Dr Giorgio Bronzetti (Laboratorio di Mutagenesi e Differenziamento, Pisa, Italy), and the assay was performed as previously described by Zimmermann (1984) and Moretton et al. (1990). Prior to each experiment the *S. cerevisiae* D7 strain was tested for the frequency of spontaneous revertants at the tryptophan (trp) locus. Cells of a stationary phase culture were treated with the samples and incubated 2 and 24 h at 28°C. After treatment washed cells suspensions were plated on appropriate media. For all the assays the data were analyzed using the modified 2-fold rule (Chu et al. 1981) in which a response is considered positive if the average response was more than twice the spontaneous frequencies. The data obtained were also subjected to an analysis of variance (Sokal and Rohlf 1994) with computer assistance (STATISTICA 5.0)

The *Bacillus subtilis* rec strains were kindly supplied by Dr. G. Mazza (Universitá di Pavia, Italy). The rec assay was performed by the determination of the efficiency of plating in presence of increasing sample concentration (Mazza 1982). To describe the genotoxic potential of a sample the data obtained were subjected to an analysis of variance (Sokal and Rohlf 1994) with computer assistance (STATISTICA 5.0)

RESULTS AND DISCUSION

The results obtained when samples of raw and treated radiographic wastewater, and the corresponding XAD extracts, where assayed in the Ames test are shown in Table 1. A considerable number of samples showed cytotoxicity for the *Salmonella* strain employed in the tests, important dilutions were necessary in order to found the limits imposed by the toxicity of the sample.

Table 1. Mutagenicity of radiographic wastewater samples in the Ames test

	(mL/plate)	*S. typhimurium* TA98* -S9	+S9	*S. typhimurium* TA100* -S9	+S9
NaCl 0,9%	10^{-1}	28 ± 5	34 ± 4	195 ± 7	104±11
Raw wastewater samples	10^{-1}	Tox		Tox	
	10^{-2}	Tox		Tox	
	10^{-4}	Tox		Tox	
	10^{-5}	22 ± 10	57±8	153 ± 9	128± 16
Treated wastewater samples	10^{-1}	29 ± 3	43±5	207 ± 17	145±23
DMSO	10^{-1}	27 ± 5	29±5	195 ± 6	136 ± 16
XAD extract raw wastewater	10^{-1}	Tox		Tox	
	10^{-2}	Tox		Tox	
	10^{-5}	14 ± 2	20±6	217 ± 20	118 ± 11
	10^{-6}	18 ± 2	25±3	171 ± 7	134 ± 19
XAD extract treated wastewater	10^{-1}	24 ± 4	33±2	156 ± 25	136 ± 10
	10^{-2}	21 ± 5	31±5	113 ± 7	110 ± 11

*Results are expressed as mean of *Salmonella* revertant colonies per plate ± standard deviation of three replicate plates. The assay was performed with (+S9) and without (-S9) microsomal activation. Tox: toxic for the *Salmonella* strains.

Follow the criteria of Mortelmans and Zeiger (2000) the results obtained from such samples were considered not mutagenic.

The dilution of the raw effluent would generate an extremely low (below the sensitivity level of the Ames test) concentration of mutagenic constituents in the aqueous fraction of the wastewater yielding negative results, both in presence and absence of microsomal activation.

Amberlite XAD resins have been the most applied method to extract organic toxicants from water samples (Kummrow et al. 2003). The reversion pattern of Ames tester strains treated with XAD 100X concentrate of radiographic waste waters is shown in Table 1. On the other hand the extract of the treated waste water, resulted less toxic and no genotoxic effect was detected either with the undiluted extract sample.

Table 2.Gene conversion frequency induced by radiographic wastewater samples and XAD extracts in *S. cerevisiae* D7

mL/100mL	GC* after 2 h incubation	GC* after 24 h incubation
Raw wastewater samples		
0.00	7.95 ± 1.53	10.68 ± 0.89
1.00	8.01 ± 0.89	9.14 ± 2.07
0.10	7.53 ± 1,65	15.51 ± 1.99
0.01	8.03 ± 1.54	11.86 ± 0.52
Treated wastewater samples		
0,00	5.77 ± 0.68	11.01 ± 1.09
100.00	8.07 ± 0.70	21.63 ± 4.36
50.00	6,57 ± 1,67	7.64 ± 0.53
10.00	7.58 ± 0.82	8.01 ± 098
XAD extract raw wastewater		
0.00	6.85 ± 0.39	10.90 ± 1.39
1.00	6.77 ± 1.71	Tox.
0.10	7.66 ± 1,67	Tox.
0.01	7.57 ± 1.59	**136.78 ± 5.72**
XAD extract treated wastewater		
0.00	12.51 ± 1,10	21.82 ± 2.90
10.00	8.07 ± 0.70	Tox.
5.00	15.26 ± 2.01	15.97 ± 3.47
1.00	7.80 ± 1.18	36.30 ± 6.20

*Gene Conversion frequency at the *trp5* locus x 10^5. The mean and standard deviations of three independent experiments are shown. Significant mutagenic responses ($p<0.05$) are indicated in bold. Tox: cytotoxicty

The results obtained with Ames test seem to indicate that the CTyT compact unit actually removes the cytotoxic load of the radiographic effluents resulting in a non genotoxic treated waste water.

The potential of raw and processed samples to induce genome rearrangements was investigated by the use of the eukaryotic yeast *S. cerevisiae* D7 strain. The results obtained for the induction of gene conversion with wastewater samples (Table 2) were similar to those obtained with the Ames test. The raw water sample showed a cytotoxic response for the yeast strain and the test was only possible with concentrations of 1.00, 0.10 and 0.01 mL/100mL.

The water samples processed through passage by CTyT compact unit were less cytotoxic and undiluted sample test were possible. No genotoxic response was found. On the other hand XAD extracts obtained from raw water was found to significantly induce gene conversion.

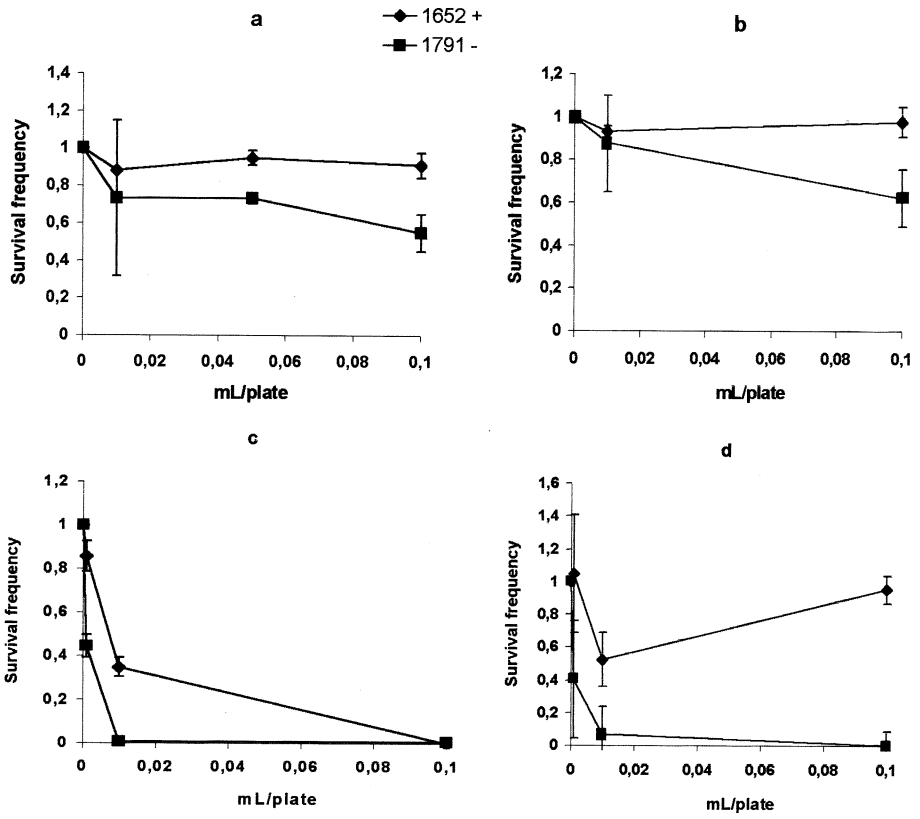

Figure 1. Survival curves for 1652 rec+ and 1791 rec- strains of *B. subtilis* tested with: **a,** raw wastewater samples; **b,** treated wastewater samples; **c,** XAD extract of raw wastewater and **d,** XAD extract of treated wastewater

In the XAD raw water extract the higher concentrations used were cytotoxic and the *S. cerevisiae* test did not allow the determination of gene conversion frequencies. When a concentration of 0.01 mL/100mL of this toxic extract was incubated during 24 h the mutation frequency found was a 12-fold increase over the spontaneous background. In the processed water extract, the maximum gene conversion frequency found did not show a significant increase over the spontaneous background (Table 2). These results indicate that the compounds responsible of the genotoxic activity, that involves partially the DNA repair of extract-induced premutagenic lesions, are removed by the passage through the CTyT unit.

In order to confirm the genotoxicity a *Bacillus subtilis* rec assay was performed. The results indicate that the CTyT filter treatment reduces the XAD extract radiographic effluent toxicity. Toxic substances are removed a few orders of magnitude during the treatment. However a positive DNA damage of the *B. subtilis* was detected (Figure 1 d).The difference between the survival curves for 1652 rec+ and 1791 rec- strains of *B. subtilis* was found extremely significant ($p<0.05$) when XAD extract of treated wastewater was tested. These results indicate that part of the genotoxic activity that involves partially the DNA repair of extract-induced premutagenic lesions, is preserved during the passage through the CTyT unit. These results complement those found with *S. cerevisiae* D7 test. Both assays detect agents that react or interact with the DNA to produce injuries recognized by specific cellular repair systems. The nature of the compounds that interact with the DNA in the complex mixture generated in the XAD extract is still unknown.

The radiographic wastewater disposal using the CTyT unit, as well as the dumping of this treated waters into the municipal sewage system, seems not to pose an obvious genotoxic pollution hazard. The radiographic effluent is diluted in the municipal wastewater to a degree that its genotoxic activity is no longer detectable in our battery test, unless the samples were submitted to an XAD concentration. However it does not mean that the genotoxicity is lost. It may still be accumulated in one of the environmental compartments and there create long term ecological effects. Therefore it seems necessary to clarify whether a group of organic compounds contribute to the genotoxic potential of radiographic wastewater. Biodegradability and persistence of the main identified compounds will have to be also analyzed to judge the impact on the environment.

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