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Influence of photodegradation on mutagenic activity of aquatic solution of chlorophenols*

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ABSTRACT

The paper presents the effect of UV radiation of 254 nm wavelength on the mutagenic activity of aquatic solutions of 3-chlorophenol (3-CP), 2,4-dichlorophenol (PCP), 2,4,6-trichlorophenol (2,4,6-TCP) and pentachlorophenol (PCP). Mutagenicity studies were carried out on two *Salmonella* strains: TA98 and TA100, with and without metabolic activation by S9 mix. Stable intermediate products of photodegradation have been identified using the GC/MS method. Chlorophenols in doses below 0.1 μg were strongly mutagenic towards both strains: TA98 and TA100. The mutagenic properties of aquatic solutions of chlorophenols for both strains decreased as follows: PCP≥2,4-DCP>2,4,6-TCP>3-CP. Aquatic solutions of three chlorophenols after their photodegradation were non-mutagenic for both bacterial strains, except for 2,4-dichlorophenol. In the case of this compound, the products of its decomposition were more mutagenic for the TA100 strain. No significant metabolic activation by S9 mix was observed which suggests a direct mutagenic impact of chlorophenols on the tested *Salmonella* strains.

INTRODUCTION

Chlorophenols belong to a group of compounds which are especially noxious to the natural environment (Hattula and Knuutinen 1985; Saito and Shigeoka 1994). They are used as intermediates in the production of dyes and chlorinated pesticides (EHC 93, 1987). Due to its biocidal properties, 4-chlorophenol is also used as a dental antiseptic (Ohara et al. 1993)

Contaminated food intake and the chlorination of both drinking water and waste water are the environmental sources of human exposure to chlorophenols. Waste containing chlorophenol may cause contamination of groundwater and the subsequent introduction of chlorophenol into drinking water supplies. Dermal exposure can occur in occupational settings. Much lower levels of dermal exposure can occur during showering and bathing with water containing chlorophenols. Formation of chlorophenols can also happen under natural chlorination processes, such as reactions between humic acid and soil microbes (Czaplicka 2004).

The World Health Organization (WHO) qualified some of the chlorophenols (i.e. 2,4,6-trichlorophenol; 2,4,5-trichlorophenol and pentachlorophenol) as compounds suspected of having carcinogenic properties. The International Agency for Research on Cancers (IARC) classified five chlorophenols: pentachlorophenol (PCP); 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP); 2,4,6-trichlorophenol (2,4,6-TCP); 2,4,5-trichlorophenol (2,4,5-TCP) and 2,4-dichlorophenol (2,4-DCP) as belonging to the 2B group of potential human carcinogens. This category encompasses those chemical agents for which there is sufficient evidence of carcinogenicity in animals and inadequate evidence of carcinogenicity in humans. Some studies suggest that chlorophenols, rather than being initiators, may be tumour promoters (IARC 1987).

Photodegradation is potentially an important process for the transformation of chlorophenols to lower chlorinated compounds (relatively less toxic) or other degradation products. The photochemical treatment of drinking water is now at the commercial level. On the other hand photodegradation of higher chlorinated phenols may lead to the formation of highly toxic products such as polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Vollmuth et al. 1994).

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2-chlorophenol (2-CP) and 4-chlorophenol (4-CP) have been tested in several genotoxicity assays, but data for 3-chlorophenol (3-CP) do not exist. The results of prokaryotic genotoxicity assays for 2-CP and 4-CP were primarily negative for their mutagenicity. In standard *Salmonella* assays treatment generally did not cause any increase in the number of revertants (DeMarini et al. 1990; Rapson et al. 1980). In one study, 4-CP had a marginally positive response in strain TA1537 (Seuferer et al. 1979) and induced an increase in number of revertants of strains: TA97, TA98, TAl00, and TA104 (Strobe and Grummt 1987). In mammalian *in vitro* systems, 2-CP induced a slight or moderate increase in c-mitosis and aneuploidy in cultured Chinese hamster lung cells (Onfelt 1987).

2,4-dichlorophenol (2,4-DCP) was mostly negative for mutagenic activity in *Salmonella* assays (Probst et al. 1981; Rapson et al. 1980; Rasanen et al. 1977) but was positive in a prophage induction assay (DeMarini et al. 1990) and positive without activation in UMU test system (One et al. 1992). In Chinese hamster V79 cells 2,4-DCP was negative for gene mutation (Hattula and Knuutinen 1985) but produced chromosomal aberrations (Onfelt 1987) and induced unscheduled DNA synthesis in rat hepatocytes (Probst et al. 1981).

2,4,6-trichlorophenol (2,4,6-TCP) has been evaluated for genotoxicity in a variety of *in vitro* assays. The reported results of these various assays have been both positive and negative, with the majority of studies reporting negative results. *In vitro*, 2,4,6-TCP has demonstrated genotoxic activity without metabolic activation in *Bacillus subtilis*, *Saccharomyces cerevisiae*, and mammalian cells (Chinese hamster V79 cells, mouse lymphoma L5178Y TK +/- cells) (Fahrig et al. 1978; Hattula and Knuutinen 1985; Kinae et al. 1981; McGregor et al. 1988). Positive results for mutations in Chinese hamster V79 cells reported by Hattula and Knuutinen (1985) are in contrast to the negative results reported by Jansson and Jansson (1992). Negative results were also observed in the case of bacteria (*Salmonella typhimurium* without activation), yeast (*Saccharomyces cerevisiae*), and in the case of mammalian ovary cells (Fahrig et al. 1978; Kinae et al. 1981; Rasanen et al. 1977).

Investigations on the genotoxic activity of pentachlorophenol (PCP) have given rise to divergent results, which would seem to make an evaluation difficult. PCP does not seem to induce gene (point) mutations, as in most bacterial assays (Seiler 1991). PCP has been reported to induce mutation in *S. typhimurium* in only 2 studies using a liquid pre-incubation protocol by Nishimura and Oshima (1983) and Nishimura et al. (1982). DeMarini et al. (1990) have shown that PCP has prophage-inducing properties in *Escherichia coli* with metabolic activation. Extensive testing in Chinese hamster CHO cells for the induction of chromosome aberration (CA) and sister chromatid exchange (SCE) yielded ambiguous results, which were summarised (Galloway et al. 1987; McConnell 1989) as constituting a "weakly positive" response.

In this paper the change in the mutagenicity of aquatic solutions of four chlorophenols: 3-chlorophenol; 2,4-dichlorophenol; 2,4,6-trichlorophenol and pentachlorophenol during UV radiation is presented. The studies were performed using the standard *Salmonella* reverse mutation plate incorporation assay with two strains: TA98 and TA100 (±S9 mix).

MATERIAL AND METHODS

Materials

3-chlorophenol; 2,4-dichlorophenol; 2,4,6-trichlorophenol and pentachlorophenol were purchased from Aldrich. D-biotin and L-histidine-HCl were obtained from Sigma (Germany). Nicotinamide adenine dinucleotide phosphate disodium salt (NADP) and glucose-6-phosphate disodium salt (G-6-P) were purchased from Fluka (Germany). Purified Agar No. 1 came from Oxoid Ltd. (Basingstoke, U.K.) and Nutrient Broth from Becton Dickinson (U.S.A.).

4-Nitroquinoline N-oxide (NQNO) was obtained from Sigma (Germany) and benzo(*a*)pyrene (B(*a*)P) from Fluka (Germany).

UV irradiation

Standard mixtures of each chlorophenol: 3-chlorophenol (3-CP); 2,4-dichlorophenol (2,4-DCP); 2,4,6-trichlorophenol (2,4,6-TCP) and pentachlorophenol (PCP), were dissolved in methanol/redistilled water mixture (5:100 v/v). Concentrations of chlorophenols in standard mixtures are given in Table 1. The solutions were placed in closed quartz glass vessels and exposed to UV radiation over 3 hours. The irradiation was carried out by a wavelength of 254 nm by 2 hours. The temperature of the solutions was 25°C.

Table 1. Concentration of chlorophenols in a standard mixture before (A) and after (B) irradiation.

Compound	Concentration, mol·l^{-1}	
	A	B
3-chlorophenol	0.04750	0.02465
2,4-dichlorophenol	1.234×10^{-3}	0.469×10^{-3}
2,4,6-trichlorophenol	1.036×10^{-3}	0.308×10^{-3}
Pentachlorophenol	0.767×10^{-3}	0.345×10^{-3}

Quantitative and qualitative analysis

The solutions after irradiation were analysed by GC/MS method. Chlorophenols isolating procedure has been described by Czaplicka (2001). Quantitative and qualitative analyses were performed by the gas chromatography method combined with a mass detector (GC/MS) using Perkin Elmer Clarus 500 gas chromatograph equipped with a DB-5 MS capillary column.

Qualitative analysis was performed comparing the retention times and the mass spectra of standards for the compounds corresponding to the particular peaks in the chromatogram with the mass spectra found in reference libraries.

Quantitative analysis was performed using a selected ion monitoring method (SIM), choosing two or three ions typical for each compound.

Mutagenic test

The *Salmonella* assay measures $his^- \rightarrow his^+$ reversion induced by chemicals that cause mutation in a gene of a histidine-requiring strain to produce a histidine independent strain of this organism. TA98 detects mutagens that cause frameshift mutations and TA100-base pair substitutions.

A plate incorporation test was performed according to the revised methods of Maron and Ames (1983). All sample doses were applied on duplicate plates, with and without additions of S9 mixture. The test samples were added to the test-tubes containing 2 ml of molten top agar (0.6% agar, 0.5% NaCl, 0.04 mM L-histidine, 0.5 mM biotin). The test samples (400 μl) consisted of 100 μl of an overnight broth culture of the bacterial tester strain and 0.5 ml of the S9 mix. S9 solution contained 8 mM $MgCl_2$, 33 mM KCl, 5 mM G-6-P, 4 mM NADP, and 100 mM sodium phosphate (pH=7.4). The S9 level routinely used was 50 μl/plate corresponding to 3 mg of protein/plate (Bradford 1976). The mixture was stirred, poured immediately onto plates of minimal agar and incubated at 37°C. The plates were incubated for 48 hrs and then histidine-independent revertant colonies together with the spontaneous revertants were counted with the use of an automatic colony counter. Whenever necessary, the numbers of revertants in positive controls (with diagnostic mutagens) and in negative controls (blank samples) were checked.

According to the Organisation for Economic Co-operation and Development (OECD), there are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate. To select the doses with linear dose vs. response relationship, a method of point rejection was applied (Bernstein et al. 1982). The mutation effect was expressed also as mutagenic rate (MR per each dose). MR is a ratio of the number of induced revertants to the number of revertant colonies in the appropriate negative control. The sample was considered evidently mutagenic when its mutagenic rate (MR) was ≥2.

Table 2. Photodegradation products identified in solution after irradiation.

Solution of:	Identified product
3-chlorophenol	Resorcinol
	Phenol
	Chlorohydroxybiphenyl
2,4-dichlorophenol	2-chlorophenol
	4-chlorophenol
	2-chlorohydroquinone
	4-chlorocatechol
2,4,6-trichlorophenol	2,4-dichlorophenol
	2,6-dichlorophenol
	4-chlorophenol
	2,4-dichlorocatechol
	2,6-dichlorohydroquinone
Pentachlorophenol	Tetrachlorobenzoquinone
	Tetrachlorocatechol
	2,3,5,6-tetrachlorophenol
	2,3,4,5-tetrachlorophenol
	2,3,5-trichlorophenol
	3,4,5-trichlorophenol
	3,5-dichlorophenol

RESULTS

Photodegradation of tested chlorophenols

After irradiating solutions by 254 nm wavelength, the concentration of each tested chlorophenol was reduced from 62% to 50% (Table 1). A qualitative analysis of samples after photodegradation showed the presence of chlorophenols and various products of their photodegradation (Table 2).

Mutagenicity of tested chlorophenols

Spontaneous reversion rates for strains were in a typical range in accordance with the literature (TA98: 37.0±3.0, TA100: 199.7±25.6). The mean number of TA98 revertants induced by NQNO was high (451 rev·plate^{-1}) in comparison with 292 rev·plate^{-1} presented by Maron and Ames (1983). It indicated that our strain TA98 was more sensitive to direct mutagens. B(*a*)P produced 103 revertant colonies of TA98 just as in methodological papers (143 rev·plate^{-1}). TA100 was about three times less sensitive both to NQNO (1298 *vs.* 4220) and B(*a*)P (557 *vs.* 937). The influence of a methanol/water mixture, in which chlorophenols were dissolved, on a number of revertant colonies was also studied. The mean number of spontaneous revertant colonies and revertants in appropriate concurrent negative controls (K1-mixture of methanol in water and K2-mixture of methanol in water and S9 mix) were similar to SRR (for TA98: 37 *vs* 38.7 *vs* 34.3; for TA100: 199.3 *vs* 240.7 *vs* 193.7).

According to Rapson et al. (1980) in the first experiments 5 doses of four chlorophenols, ranging from 0.1 to 1000 μg per plate, were tested using strains TA98 and TA100, with and without metabolic activation.

The research carried out on chlorophenol solutions before irradiation showed that even the lowest dose (0.1 μg) was very toxic for both bacterial strains with and without metabolic activation. For that reason the correct conclusions on both the mutagenic activity of pure compounds and the influence of irradiation on the level of its mutagenicity cannot be drawn. Therefore, in the second set of tests three doses corresponding to 0.0008, 0.008 and 0.08 μg of the compound per plate were examined. The mutagenic tests were done for 3-chlorophenol; 2,4-dichlorophenol; 2,4,6-trichlorophenol and pentachlorophenol solutions before and after irradiation.

TA98 strain

Due to there being no evidence of a significant influence of S9-mix on mutagenicity of all the tested chlorophenols, we presented only the result of the direct acting variant of experiments. Regarding the TA98 strain, any significant linear correlation between the three tested doses of all chlorophenols and induced number of revertants were stated. Only 2,4,6-trichlorophenol before irradiation showed a linear relationship between the dose and the number of revertants ($p<0.000$) (but only after rejection of the results obtained for 0.08 μg dose, in which case a significant decrease in numbers of induced revertants was observed). The differences between the mean number of revertants in the appropriate negative control and the number of revertants induced by successive doses of the examined compounds before irradiation and in the mixture after irradiation were evaluated using one-factor analysis of variance (ANOVA). All the tested doses of chlorophenols were mutagenic toward the strain TA98. Also the highest number of revertants was obtained for various doses (Figure 1).

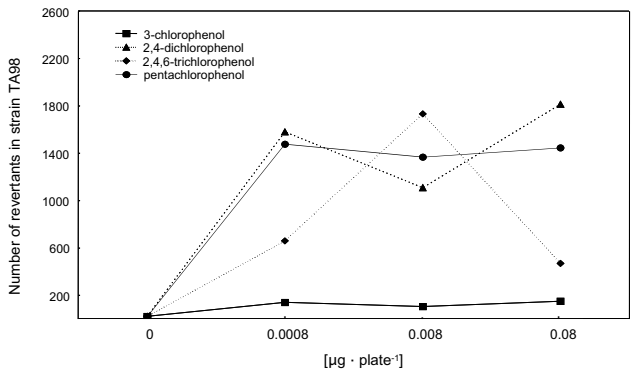

Figure 1. Mutagenicity of 3-chlorophenol; 2,4-dichlorophenol; 2,4,6-trichlorophenol and pentachlorophenol for TA98(-S9) before irradiation.

For each tested dose a mutagenic activity was calculated. The results obtained indicate the highest mutagenicity of PCP and 2,4-DCP for the TA98 strain (frameshift mutation), and the lowest one as caused by 3-chlorophenol (Table 3). Products formed during photodegradation, which derived from examined chlorophenols, did not reveal their mutagenic properties for the TA98 strain.

Table 3. Mutagenic rate (MR) caused by different doses of tested chlorophenols applied before (A) and after (B) their irradiation towards the strain TA98-S9.

Dose [μg·plate^{-1}]	3-CP		2,4-DCP		2,4,6-TCP		PCP	
	A	B	A	B	A	B	A	B
0.0008	5.03	0.07	66.8	0.03	27.3	-0.31	62.3	0.07
0.008	3.53	-0.29	46.6	0.14	73.4	-0.12	57.7	-0.29
0.08	5.44	0.16	76.8	0.22	19.2	0.31	61.1	0.37

TA100 strain

Linear correlation was obtained only for 3-chlorophenol, both before (p=0.013) and after irradiation (p=0.004). In the case of 3-CP; 2,4,6-TCP and PCP no significant influence of S9-mix was observed. The differences between the mean number in appropriate control and successive doses of the examined compound before irradiation were evaluated by ANOVA (Figure 2).

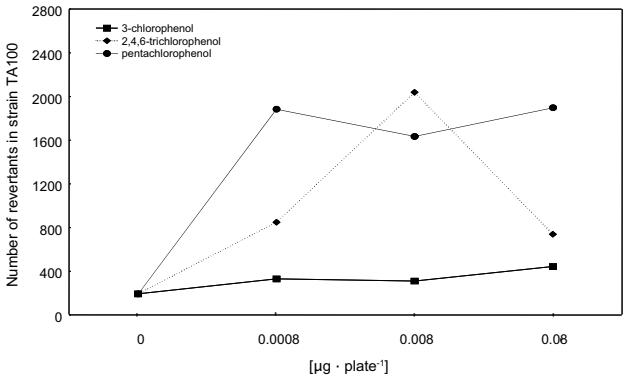

Figure 2. Mutagenicity of 3-chlorophenol, 2,4,6-trichlorophenol and pentachlorophenol for TA100-S9 before irradiation.

2,4,6-trichlorophenol and pentachlorophenol were mutagenic for the strain TA100 although the highest frequency of induced revertants were stated in various doses: 2,4,6-TCP in 0.008 μg and PCP in 0.0008 μg, respectively. Chlorophenol did not demonstrate any mutagenic property toward this strain.

The values of mutagenic rate calculated for each tested dose (Table 4) confirmed the highest mutagenicity of pentachlorophenol and 2,4,6-trichlorophenol for the TA100 strain (base substitution mutation), and the lowest one as caused by 3-chlorophenol. Nevertheless, MR values were significantly lower in comparison with the TA98 strain. The mixtures of three chlorophenols after photoreaction did not reveal a mutagenic effect on cells of TA100.

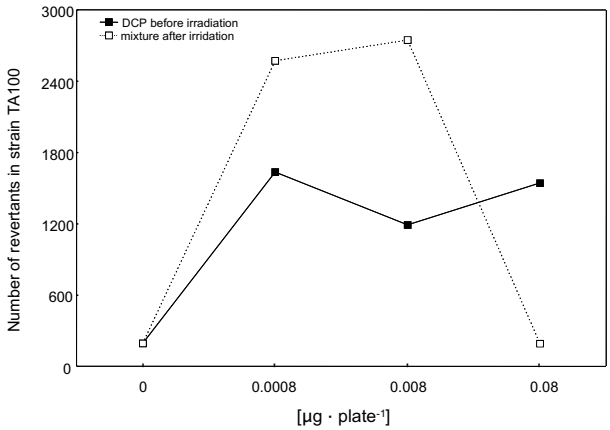

Figure 3. Mutagenicity of 2,4-dichlorophenol for TA100-S9, before and after irradiation.

A different mutagenic mechanism of 2,4-dichlorophenol was observed in the case of TA100 strain (Figure 3). Aquatic solutions of 2,4-DCP before irradiation were mutagenic in the range of tested doses, but the mixtures after reaction induced a significantly stronger mutagenic effect than the initial solutions in two doses: 0.0008 and 0.008 μg. In the highest dose the photoproducts showed a strong toxic effect for strain TA100. Similarly, as in the case of the TA98 strain, the S9 influence was not stated, which also suggested a direct effect of 2,4-DCP on this strain.

DISCUSSION

Although there is sufficient evidence for the carcinogenicity of several of the isomers of chlorophenol, these agents do not appear to induce point mutation in *Salmonella* bacteria. 2,4-DCP and 2,4,6-TCP, tested over a wide range of concentrations (0.5, 5, 50 and 500 μg·plate^{-1}), caused no significant increase in revertant colonies of TA98 (± S9) and TA100 (± S9) (Rasanen et al. 1977). Rapson et al. (1980) studied the mutagenicity of 93 pure substances used in chlorinating experiments. Three chlorophenols: 3-CP; 2,4-DPC and

Table 4. Mutagenic rate (MR) caused by different doses of three tested chlorophenols applied before (A) and after (B) their irradiation towards the TA100-S9.

Dose [μg·plate^{-1}]	3-CP		2,4-DCP		2,4,6-TCP		PCP	
	A	B	A	B	A	B	A	B
0.0008	0.71	0.03	7.44	12.28	3.38	1.97	8.72	0.02
0.008	0.60	0.07	5.15	13.18	9.52	0.00	7.43	0.04
0.08	1.29	0.14	6.97	0.01	2.80	0.04	8.49	0.04

2,4,6-TCP tested in five doses (0.1, 1, 10, 100 and 1000 $\mu g \cdot plate^{-1}$) have no mutagenic properties towards the strain TA100, in both variants of metabolic activation. In a standard *Salmonella* assay with the set of strains: TA98, TA100, TA1535, TA1537 and TA1538, treatment of 3-CP (10-1000 $\mu g \cdot plate^{-1}$); 2,4-DCP (3,3-333 $\mu g \cdot plate^{-1}$); 2,4,6-TCP (10-666.7 $\mu g \cdot plate^{-1}$) and PCP (0.3-30 $\mu g \cdot plate^{-1}$) did not produce an increased number of revertants (Havorth et al. 1983). Negative findings occurred both in the presence and absence of their metabolic activation.

The results of the study are different from those published in earlier papers. In this study it was found that all the tested chlorophenols in doses above 1 $\mu g \cdot plate^{-1}$ were very toxic for both *Salmonella* strains, TA98 and TA100. When the much lower doses, ranging from 0.0008 to 0.08 $\mu g \cdot plate^{-1}$, were tested it was found that aqua solutions of pure 2,4-DCP; 2,4,6-TCP and PCP were very mutagenic towards TA98 and TA100, regardless of metabolic activation.

The results obtained indicated the high mutagenicity of PCP; 2,4-DCP and 2,4,6-TCP for TA98 strain (frameshift mutation), and the lowest one caused by 3-CP. Products formed during photodegradation, derived from examined chlorophenols, did not reveal their mutagenic properties for TA98 strain. No observed influence of metabolic activation by S9-mix indicated that the tested chlorophenols directly affected mutation in the tested strain.

PCP and 2,4-DCP were the most mutagenic for the TA100 strain, as in the case of TA98. On the other hand, any effect of 3-CP for this strain was stated. Mutagenicity of the mixture after reaction resulting from irradiation of 2,4-dichlorophenol solution was higher than the mutagenicity of 2,4-DCP, with and without metabolic activation. This suggests that compounds which were formed during this process are characterized by a higher mutagenic activity of the type "base substitution".

Photodegradation caused a slight decrease of mutagenic activity of 2,4,6-TCP for TA100 strain, while the products of photochemical decomposition of PCP in the aquatic environment did not show the mutagenic effect for this strain. Similarly to the TA98 strain, any significant influence of S9-mix on mutagenic effect was stated which indicates a direct mutagenic effect of examined chlorophenols and their photo-derivatives for TA100 strain.

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