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Sodium hypochlorite-, chlorine dioxide- and peracetic acid-induced genotoxicity detected by the Comet assay and *Saccharomyces cerevisiae* D7 tests

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Mutagenicity of drinking water is due not only to industrial, agricultural and urban pollution but also to chlorine disinfection by-products. Furthermore, residual disinfection is used to provide a partial safeguard against low level contamination and bacterial re-growth within the distribution system. The aims of this study were to further evaluate the genotoxic potential of the world wide used disinfectants sodium hypochlorite and chlorine dioxide in human leukocytes by the Comet assay and in *Saccharomyces cerevisiae* strain D7 (mitotic gene conversion, point mutation and mitochondrial DNA mutability, with and without endogenous metabolic activation) and to compare their effects with those of peracetic acid, proposed as an alternative disinfectant. All three disinfectants are weakly genotoxic in human leukocytes (lowest effective dose 0.2 p.p.m. for chlorine dioxide, 0.5 p.p.m. for sodium hypochlorite and peracetic acid). The results in *S.cerevisiae* show a genotoxic response on the end-points considered with an effect only at doses higher (5- to 10-fold) than the concentration normally used for water disinfection; sodium hypochlorite and peracetic acid are able to induce genotoxic effects without endogenous metabolic activation (in stationary phase cells) whereas chlorine dioxide is effective in growing cells. The Comet assay was more sensitive than the yeast tests, with effective doses in the range normally used for water disinfection processes. The biological effectiveness of the three disinfectants on *S.cerevisiae* proved to be strictly dependent on cell-specific physiological/biochemical conditions. All the compounds appear to act on the DNA and peracetic acid shows effectiveness similar to sodium hypochlorite and chlorine dioxide.

Introduction

The disinfection process is an effective barrier to many pathogens in drinking water. However, many studies have detected the presence of mutagens in drinking water, due not only to different pollution sources but also to disinfection treatment (Kraybill, 1981; Miller *et al*., 1986; Meier, 1988; Vartiainen *et al*., 1988; Monarca and Pasquini, 1989; Peters *et al*., 1990; Suzuki and Nakanishi, 1990; Kusamram *et al*., 1994; Filipic *et al*., 1995; Sabouni and Zia'ee, 1995; Rehena *et al*., 1996; Cantor, 1997; Hofer and Shukes, 2000). It is known that chlorine reacts with organic matter (humic and fulvic acids derived from plant decomposition) present in untreated surface water to give disinfection by-products (Kruithof, 1985; Monarca *et al*., 1985; Cognet *et al*., 1986; Kowbel *et al*., 1986; Fielding and Horth, 1986; Richardson *et al*., 1994; De Marini *et al*., 1995; Lee *et al*., 2001; Woo *et al*., 2002). Furthermore, residual disinfection is used to provide a partial safeguard against low level contamination and re-growth within the distribution system. Consequently, disinfectants are present in tap water together with disinfection by-products. Even if the exposure level is low, the presence of these compounds in drinking water must be taken into account because of lifelong exposition.

Chlorine provides very effective protection against waterborne infectious disease, but there are some epidemiological reports of a cancer risk associated with chlorinated water (Meier, 1988; Flaten, 1992; Morris, 1995). Problems associated with chlorine [as hypochlorous acid (HOCl) and hypochlorite ion (OCl^-)] as a water disinfectant have occurred because of the formation of trihalomethanes (THMs) and other potentially mutagenic/carcinogenic reaction by-products. The genotoxic activity and carcinogenic action of chlorinated drinking water are also considered to be due to OCl^- itself (Whiteman *et al*., 1999; Ohnishi *et al*., 2002). However, NaClO is considered non-classifiable for carcinogenicity by the IARC (1991) (group 3), although it was shown to give positive results (Meier *et al*., 1985; Wlodkowski and Rosenkranz, 1975; Ishidate *et al*., 1984; Ishidate, 1988) in some short-term mutagenicity tests: *Escherichia coli* and *Salmonella typhimurium* (point mutation), Chinese hamster (chromosome aberration), human fibroblasts (sister chromatid exchange) and mouse (sperm morphology). Furthermore, OCl^- was shown to directly induce different kinds of DNA damage in relation to the cellular content (Patton *et al*., 1972; Winterbourn, 1985; Whiteman *et al*., 1997, 1999; Ohnishi *et al*., 2002).

The increasing use of ClO_2 for drinking water treatment has warranted the need to monitor the levels of possible hazardous by-products, including chlorite (ClO_2^-) and chlorate (ClO_3^-) (Environmental Protection Agency, 1994). ClO_2 is considered to be a more effective bactericide than aqueous chlorine and it reduces the formation of chlorinated organics. Neither ClO_2 nor its by-products, ClO_2^- and ClO_3^-, appear to react with humic or fulvic acids to form THMs, as does chlorine in drinking water treatment (Miltner, 1977). However, ClO_2 is a strong oxidant that reacts with organic material to produce a variety of oxidized by-products (Tan *et al*., 1987; Kim *et al*., 1999). The US Environmental Protection Agency (1996) classified ClO_2 in group D, i.e. non-classifiable for human carcinogenicity, due to the inadequacies of the human and animal data and because the data on ClO_2 genotoxicity are still uncertain and have been the subject of considerable controversy (Ishidate *et al*., 1984; Meier *et al*., 1985; Tan *et al*., 1987; Hayashi *et al*., 1988; Kim *et al*., 1999; Environmental Protection Agency, 2000).

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Table I. Cytotoxic and genotoxic effects in *Saccharomyces cerevisiae* D7 strain

	Sample	Dose	–P450				+P450			
			Cell survival (%)	*trp5* locus ($\times10^{-8}$ cells)	*ilv1* locus ($\times10^{-8}$ cells)	RD ($\times10^{-3}$ cells)	Cell survival (%)	*trp5* locus ($\times10^{-8}$ cells)	*ilv1* locus ($\times10^{-8}$ cells)	RD ($\times10^{-3}$ cells)
A	EMS	100 mM	94 ± 3	68630 ± 17140	17487 ± 1677					
	2AF	5 µg/ml	97 ± 2	930 ± 110	42 ± 7		92 ± 7	28590 ± 3230	3129 ± 482	
	EtBr	1 µg/ml	95 ± 6			134 ± 12				196 ± 17
B	NaClO	0 p.p.m.	100	917 ± 146	34 ± 4	4 ± 1	100	1767 ± 227	32 ± 5	3 ± 2
		0.2 p.p.m.	85 ± 5	945 ± 90	27 ± 7	3 ± 2	91 ± 5	2125 ± 215	35 ± 4	3 ± 1
		1 p.p.m.	77 ± 4[a]	809 ± 206	30 ± 2	5 ± 3	92 ± 5	2133 ± 193	42 ± 15	4 ± 1
		5 p.p.m.	67 ± 3[c]	960 ± 471	32 ± 4	6 ± 4	84 ± 4[a]	1610 ± 110	31 ± 8	5 ± 1
		10 p.p.m.	53 ± 2[c]	942 ± 261	59 ± 6[b]	13 ± 5	71 ± 4[c]	2032 ± 248	42 ± 1	3 ± 1
		20 p.p.m.	20 ± 2[c]	1376 ± 416	53 ± 7[a]	23 ± 8[a]	53 ± 3[c]	1683 ± 148	30 ± 5	5 ± 2
		30 p.p.m.	14 ± 1[c]	2034 ± 192[c]	63 ± 2[c]	30 ± 10[c]	44 ± 2[c]	1592 ± 49	39 ± 4	3 ± 1
C	ClO_2	0 p.p.m.	100	925 ± 155	25 ± 5	4 ± 1	100	1770 ± 170	25 ± 10	3 ± 1
		0.05 p.p.m.	94 ± 6	898 ± 272	33 ± 4	5 ± 1	95 ± 4	1724 ± 84	25 ± 10	4 ± 4
		0.1 p.p.m.	96 ± 5	725 ± 175	37 ± 4	5 ± 1	97 ± 5	1695 ± 95	22 ± 8	4 ± 2
		0.25 p.p.m.	89 ± 5	794 ± 157	37 ± 4	5 ± 1	96 ± 6	1916 ± 76	23 ± 8	3 ± 1
		0.5 p.p.m.	90 ± 4	755 ± 135	35 ± 1	4 ± 2	95 ± 4	1680 ± 420	22 ± 2	2 ± 1
		1 p.p.m.	83 ± 5[a]	910 ± 150	38 ± 5	5 ± 2	96 ± 4	1540 ± 230	26 ± 10	2 ± 2
		2 p.p.m.	71 ± 4[c]	913 ± 110	40 ± 5	5 ± 3	97 ± 5	1937 ± 77	32 ± 8	4 ± 3
		5 p.p.m.	54 ± 2[c]	865 ± 145	40 ± 3	4 ± 1	87 ± 4[b]	2107 ± 84	34 ± 9	6 ± 3
		10 p.p.m.	10 ± 1[c]	1100 ± 102	45 ± 5	3 ± 1	61 ± 2[c]	2675 ± 85[c]	89 ± 14[c]	12 ± 4[a]
D	PAA	0 p.p.m.	100	925 ± 155	25 ± 3	4 ± 1	100	1755 ± 55	22 ± 8	3 ± 2
		0.2 p.p.m.	96 ± 3	870 ± 70	34 ± 4	5 ± 1	100 ± 3	1666 ± 246	17 ± 9	3 ± 1
		0.5 p.p.m.	94 ± 5	875 ± 145	35 ± 4	5 ± 1	97 ± 5	1667 ± 404	14 ± 8	2 ± 1
		1 p.p.m.	85 ± 5	855 ± 105	37 ± 3	6 ± 2	99 ± 3	1765 ± 25	16 ± 3	5 ± 2
		2 p.p.m.	80 ± 4[b]	835 ± 65	36 ± 4	6 ± 1	96 ± 6	1660 ± 80	15 ± 5	4 ± 1
		5 p.p.m.	83 ± 3[b]	950 ± 30	58 ± 4[b]	4 ± 1	98 ± 4	1900 ± 190	18 ± 10	4 ± 3
		10 p.p.m.	52 ± 3[c]	1985 ± 45[c]	109 ± 15[c]	5 ± 3	97 ± 4	1550 ± 202	17 ± 7	3 ± 1
		15 p.p.m.	Toxic				79 ± 3[b]			

Gene conversion frequencies at the *trp5 locus*, reversion frequencies of the *ilv1-92* mutant and respiratory deficient (RD) frequencies in stationary (–P450) and logarithmic (+P450) growth phase cells are reported. (A) Positive controls: EMS, ethyl methanesulfonate; 2AF, 2-aminofluorene; EtBr, ethidium bromide. (B) Sodium hypochlorite (NaClO). (C) Chlorine dioxide (ClO_2). (D) Peracetic acid (PAA). Values represent means ± SD of at least three independent experiments.
[a]$P < 0.05$ (Dunnett's *C*).
[b]$P < 0.01$ (Dunnett's *C*).
[c]$P < 0.001$ (Dunnett's *C*).

In this context, an inter-University project was carried out to evaluate potential genotoxic health risks from water disinfection with chlorine and to consider peracetic acid (PAA) as an alternative disinfectant to chlorine. PAA is worth studying for its application in drinking water disinfection, since it is a potent antimicrobial agent and has many applications in hospitals, laboratories and factories (Baldry *et al*., 1991, 1995; Lefevre *et al*., 1992) and it has been found to be an effective biocidal compound for wastewater disinfection (Monarca *et al*., 2000). Data from PAA studies do not raise immediate concern as to mutagenicity (Monarca *et al*., 2002, 2003), carcinogenicity (Muller *et al*., 1988) or reproductive toxicity, the lethal dose on the species studied always being higher than the doses necessary for disinfection processes (http://www.hhmi.org/research/labsafe/lcss/lcss68.html).

As a first step, it seemed interesting to determine and compare the genotoxic effectiveness of the three disinfectants NaClO, ClO_2 and PAA. In the study reported here, the genotoxic effects of the disinfectants were evaluated with both a standardised 'short-term' test on *Saccharomyces cerevisiae* (Zimmermann *et al*., 1975) and with the Comet assay (Singh *et al*., 1988) on human leukocytes. The *S.cerevisiae* diploid strain D7 assay is able to demonstrate nuclear DNA effects, such as gene conversion and point mutation, and to assess mutation induction in the mitochondrial genome, i.e. respiratory proficiency (RP) to respiratory deficiency (RD). The SCGE or Comet assay is a widely used method for detecting DNA damage (strand breaks, alkali-labile sites and cross-linking) and incomplete excision repair sites in individual cells (Fairbairn *et al*., 1995; Cotelle and Ferard, 1999; Albertini *et al*., 2000; Kassie *et al*., 2000; Møller *et al*., 2000; Tice *et al*., 2000; Sasaki *et al*., 2000).

Materials and methods

Chemicals

Reagents for the Comet assay and general laboratory chemicals were from Sigma; media and agar for *S.cerevisiae* cultures were from Difco. Sodium hypochlorite (NaClO) (CAS no. 10022-70-5) was from Solvay S.p.A. (Rosignano, Italy), chlorine dioxide (ClO_2) (CAS no. 10049-04-4) from Caffaro (Brescia, Italy) and peracetic acid (CH_3COO_2H) (CAS no. 79-21-0) from Promox S.r.l. (Leggiuno, Italy).

NaClO and ClO_2 were always titrated (American Public Health Association, 1998) before use, because of their instability: NaClO by an iodometric titration and ClO_2 by a DPD method (Katalase from Merck KgaA, Darmastadt, Germany; DPD Total Chlorine Reagent Powder Pillow from Hach Co., Loveland, CO).

Bioassays

Saccharomyces cerevisiae. The diploid strain D7 of *S.cerevisiae* was used to determine the frequencies of (i) reversion at the *ilv1-92* locus, (ii) mitotic gene conversion at the *trp5* locus and (iii) petite colonies, i.e. mitochondrial DNA mutability, with or without endogenous activation. As an alternative system to the microsomal assay, yeast cells were harvested during the logarithmic phase of growth in 20% glucose at maximum activation of the cytochrome P450 complex (Rossi *et al*., 1997; Poli *et al*., 1999; Buschini *et al*., 2003). Both with and without endogenous metabolic activation (+/–P450) cells (10^8 cells/ml in 0.1 M phosphate buffer, pH 7.4) were treated with the disinfectants (in hermetically sealed test tubes) at 37°C for 2 h: 2-aminofluorene and ethyl

Table II. Cytotoxic and genotoxic effects in human leukocytes (Comet assay) induced by sodium hypochlorite (NaClO), chlorine dioxide (ClO_2) and peracetic acid (PAA)

Dose (p.p.m.)	Cell survival (%)			Median tail length (μm)		
	NaClO	ClO_2	PAA	NaClO	ClO_2	PAA
0	99 ± 2	98 ± 2	99 ± 1	7.83 ± 0.37	7.65 ± 0.32	7.77 ± 0.41
0.1	97 ± 2	97 ± 4	99 ± 1	7.93 ± 0.38	7.82 ± 0.39	7.46 ± 0.45
0.2	99 ± 1	96 ± 2	96 ± 5	7.91 ± 0.34	8.63 ± 0.38[a]	8.57 ± 0.55
0.5	97 ± 1	94 ± 4	99 ± 2	8.75 ± 0.46[a]	10.21 ± 0.51[c]	9.18 ± 0.41[b]
1	84 ± 3[c]	95 ± 4	96 ± 2	9.01 ± 0.40[c]	10.57 ± 0.53[c]	9.87 ± 0.50[c]
2			95 ± 4			10.27 ± 0.49[c]
5	79 ± 2[c]	91 ± 5	93 ± 4	10.98 ± 0.52[c]	12.45 ± 0.60[c]	10.77 ± 0.51[c]

The median tail length of the positive control (2 mM EMS) is 53.13 ± 5.71 μm. Values represent means ± SD of at least three independent experiments.
[a]$P < 0.05$ (Dunnett's *C*).
[b]$P < 0.01$ (Dunnett's *C*).
[c]$P < 0.001$ (Dunnett's *C*).

methanesulfonate were used as positive controls when cytochrome P450 was induced or not induced, respectively (Table IA). *Saccharomyces cerevisiae* does not have all the human P450 families: the identified genes are *CYP51*, also present in humans and specific for sterol biosynthesis, and *CYP56* and *CYP61*, with undetermined specificities (Nelson *et al*., 1996). However, yeast P450 efficacy in activating 2-aminofluorene, a pro-mutagenic compound widely used as a positive control in the Ames test using S9 mix, was stated in the present study (Table IA).

Mitochondrial DNA mutation induction was evaluated in the same strain by determining the frequencies of respiration-deficient colonies (RD). To detect *petite* mutants, after 5 days incubation at 28°C, the plates were overlayed with agar containing 2,3,5-triphenyl tetrazolium chloride (Ogur *et al*., 1957) for colony staining. Ethidium bromide (1 μg/ml) was used as a positive control (Table IA).

A least squares linear regression analysis was used to calculate specific genotoxic activity (mutants, convertants or RDs per p.p.m. disinfectant).

For all the assays the data (three independent experiments at least) were subjected to a variance analysis (SPSS 11). If a significant *F* value ($P < 0.05$) was obtained, the data were subjected to Dunnett's *C*-test.

Human leukocytes. For leukocyte isolation, whole blood of three healthy non-smoking donors was centrifuged in lysis buffer (155 mM NH_4Cl, 5 mM $KHCO_3$, 0.005 mM Na_2EDTA, pH 7.4), washed with phosphate-buffered saline and resuspended (~10^6 cells/ml) in RPMI 1640 medium. Aliquots (10^6 cells) were treated (1 h, 37°C) with the disinfectants or with ethyl methane sulfonate (positive control).

Cell viability was checked by fluorescein diacetate/ethidium bromide assay (Merk and Speit, 1999) and the Comet assay only performed for viabilities ⩾70%, basically according to Singh *et al*. (1988): cell lysis at 4°C overnight (2.5 M NaCl, 10 mM Na_2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10), DNA unwinding for 20 min in an electrophoretic alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, pH ⩾ 13, 0°C), electrophoresis for 20 min, (0.78 V/cm, 300 mA) at 0°C in the same buffer, followed by neutralization (0.4 M Tris–HCl, pH 7.5). DNA was stained with 100 μl of ethidium bromide (2 μg/ml) before examination at 400× magnification under a Leika DMLB fluorescent microscope (excitation filter BP 515–560 nm, barrier filter LP 580 nm) using an automatic image analysis system (Cometa Release 2.1; Sarin). The migration distance between the edge of the comet head and end of the tail (tail length, TL) provided representative data on genotoxic effects. The samples were coded and evaluated blind (50 cells for each of two replicate slides per data point). All the tests were performed at least three times.

A SPSS 11 statistical package was applied to the data of the Comet assay. Statistical differences between controls and treated samples were first determined with the non-parametric Wilcoxon rank sum test for each experiment. The median values of comet length distribution (at least three independent experiments) were used in a one-way analysis of variance test. If a significant *F* value of $P < 0.05$ was obtained, a Dunnett's *C* multiple comparison analysis was conducted.

Results

NaClO

A dose-dependent cell mortality rate was observed with 1 and 5 p.p.m. as the lowest effective doses (LED) in stationary (stat) and logarithmic (log) growth phase *S.cerevisiae* cells, respectively (Table IB). Significant genotoxic effects were only observed in stat cells for gene conversion (⩾30 p.p.m.), reversion of *ilv92* mutant (⩾10 p.p.m.) and respiration-deficient colonies (⩾20 p.p.m.). Both convertant and RD frequencies increased with disinfectant concentration, whereas revertant induction up to 10 p.p.m. appeared to be limited by the corresponding high cytotoxicity.

A significant effect ($P < 0.001$) on cell survival was detected at 1 p.p.m. (16% of cell mortality) in the Comet assay (Table II). The LED for induction of a significant increase ($P < 0.05$) in DNA migration is 0.5 p.p.m. However, the slope of the dose–response curve appears very slight (TL increase/p.p.m. = 0.061 μm, $R^2 = 0.94$).

ClO_2

Stat *S.cerevisiae* cells showed a higher sensitivity to ClO_2 toxicity than log cells, with significant effects at 1 ($P < 0.05$) and 5 p.p.m. ($P < 0.01$), respectively (Table IC). Genotoxic effects were only observed in P450-induced cells with significance only at the highest dose (gene conversion and reversion, $P < 0.001$; RD, $P < 0.05$).

No significant cytotoxic effects were observed in the Comet assay whereas a significant DNA migration increase ($P < 0.05$) was observed at 0.2 p.p.m. with a TL increase/p.p.m. = 0.082 ($R^2 = 0.75$) (Table II).

PAA

Cell mortality was heavily dependent on the physiological state of the yeast: in P450-induced *S.cerevisiae* cells a significant effect ($P < 0.01$) of PAA on cell survival was observed only at the highest dose (15 p.p.m.), whereas the LED in stat cells is 2 p.p.m. ($P < 0.01$) (Table ID). The genotoxicity was also dependent on the cellular 'state', with significant effects only in stat cells (10 p.p.m. for gene conversion; 5 p.p.m. for reversion; no RD induction).

PAA did not induce any significant cytotoxicity in the Comet assay; only genotoxic effects were observed, with a LED of 0.5 p.p.m. ($P < 0.01$) and TL increase/p.p.m. = 0.055 ($R^2 = 0.62$) (Table II).

Discussion

Drinking water quality is a health concern in countries at all levels of economic development and microbial hazards continue to be a primary worry. Therefore, disinfection is of unquestionable importance in the supply of safe drinking water. The disinfection process is an effective barrier to many

pathogens, especially bacteria, and should be used for surface waters and ground water subject to faecal contamination. Residual disinfection is used to provide a partial safeguard against low level contamination and re-growth within the distribution system. However, the use of chemical disinfectants in water treatment usually results in the formation of by-products that can be toxic and/or mutagenic (Kruithof, 1985; Monarca *et al*., 1985; Cognet *et al*., 1986; Kowbel *et al*., 1986; Fielding and Horth, 1986; Richardson *et al*., 1994; De Marini *et al*., 1995; Lee *et al*., 2001; Woo *et al*., 2002). Furthermore, the residual disinfectants themselves could be harmful, primarily due to the prolonged periods of exposure. The aim of this study was to further evaluate the potential genotoxicity induced by two widely used water disinfectants (NaClO and ClO_2) and to compare their effects with those eventually induced by peracetic acid, proposed as an alternative disinfectant.

The yeast *S.cerevisiae* is proposed as a cell model to provide rapid screening of the different biological activities of chemicals (Resnick and Cox, 2000) and to study the induction of recombination. Given the evolutionary conservation of the various DNA repair systems in eukaryotes, it is likely that the knowledge gathered about induced recombination in yeast is applicable to mammalian cells and thus to humans (Kupiec, 2000). Many carcinogens are known to induce recombination and to cause chromosomal rearrangements. An understanding of the mechanisms by which genotoxic agents cause increased levels of recombination will have important consequences for the treatment of cancer and for the assessment of risks arising from exposure to genotoxic agents in humans.

The findings on *S.cerevisiae* D7 show a genotoxic response for the end-points considered with an effect at doses higher than the disinfection standards (1–2 p.p.m. for ClO_2 and NaClO).

The genotoxic effects of NaClO without endogenous metabolic activation (in stationary phase cells) are worth noting. In fact, OCl^- itself is reported to interact with DNA to form oxidized bases (Whiteman *et al*., 1997; Ohnishi *et al*., 2002) and halogenated bases (Patton *et al*., 1972; Whiteman *et al*., 1999). The behaviour of PAA is similar to that of NaClO, with genotoxic effects induced only in stationary phase cells. Indeed, in aqueous solution it reacts to give, together with acetic acid, hydrogen peroxide, a compound able to induce DNA damage directly. The lack of effects in log yeast could be explained by the peculiar yeast metabolism. During respiration, mitochondria are the primary source of reactive oxygen species (ROS) in the cell, given that the respiratory chain of the mitochondrion is an endogenous source of ROS. However, *S.cerevisiae* growing in 20% glucose obtain their energy supply through the fermentative pathway and, consequently, could utilize antioxidant mechanisms (not used against ROS deriving from mitochondrial metabolism) to detoxify oxidant xenobiotics.

On the other hand, the genotoxicity of ClO_2 in *S.cerevisiae* is detected only with endogenous metabolic activation (in growing cells). Chlorine dioxide was found to increase reverse mutation in *S.typhimurium* with activation (Ishidate *et al*., 1984). However, water samples disinfected with chlorine dioxide did not induce reverse mutations in *S.typhimurium* with or without activation (Miller *et al*., 1986). Furthermore, studies using the *Salmonella* microsome assay of ClO_2 reaction products with some peptides (L-aspartyl-L-phenylalanine methyl ester, i.e. aspartame, L-glycyl-L-tryptophan and L-tryptophyl glycine) and amino acids (hydroxyproline and tyrosine) show mutagenic activity in the presence and absence of rat liver S9 mix (Tan *et al*., 1987). These data are uncertain and, consequently, the mechanisms of ClO_2 action remain incompletely understood.

Peracetic acid is negative in the RD assay, in both stat and log cells, in spite of the high responsiveness of mitochondrial DNA. Furthermore, the data show that treatment with ClO_2 induces only a slight increase in RD mutants ($P < 0.05$) whereas a dose–response effect is clear in cells treated with NaClO. All the disinfectants act as oxidants, but halogenated compounds such as 8-chloroadenine (Whiteman *et al*., 1999) and 4-*N*-chlorocytosine (Patton *et al*., 1972) have also been found after OCl^- exposure. The effectiveness of NaClO on mitochondrial DNA could be due mainly to the production of halogenated bases whereas the oxidative damage could be partially (ClO_2) or completely (PAA) prevented by the highly active mitochondrial defences against ROS in yeast, including Mn superoxide dismutase, cytochrome c peroxidase and glutathione peroxidase (Grant *et al*., 1997).

DNA damage, measured by the Comet assay on human leukocytes after a 1 h treatment, shows that all three disinfectants are able to induce genotoxicity (0.5 p.p.m. was the lowest effective dose for NaClO and PAA and 0.2 p.p.m. for ClO_2). Although the increase in DNA migration is small, measuring a direct end-point such as DNA damage, the effective dose values are lower in this test and in the concentration range used for water disinfection. Thus, a different sensitivity for the three compounds examined was found in the Comet assay relative to the yeast tests, with very high differences (>50-fold) in the lowest effective dose.

The choice of end-point is a determining step in genotoxicity studies, dose–effect analysis and the assessment of thresholds. Several factors, such as bioavailability, metabolic activation/inactivation, DNA repair, apoptosis and cell survival can modulate the final effect at the analysed end-point level. As shown here, these concepts are clearly evident in comparing the Comet assay, a simple system in which the end-point corresponds to the target (DNA damage), with the *S.cerevisiae* test, in which the end-point is closed but still different from the target (point mutation, gene conversion and mitochondrial mutability).

The Comet assay is more sensitive than the yeast test, possibly due to the presence of a cell wall in yeast, which might protect the cell by inhibiting uptake of the test compounds. On the other hand, the biological effectiveness of the three disinfectants on *S.cerevisiae* proved to be strictly dependent on cell-specific physiological/biochemical conditions. The data obtained from the different bioassays give a better and wider genotoxicological profile of the compounds analysed.

Comparing all the results of the study, all the compounds appear to act on DNA and the possible alternative disinfectant peracetic acid shows an effectiveness similar to sodium hypochlorite and chlorine dioxide. In this first part of an inter-University research programme the genotoxicity of pure compounds was assessed. A study of the genotoxic effects of the by-products possibly produced by the three disinfectants when mixed with humic acids is in progress.

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