MUTATION RESEARCH

Genetic Toxicology and Environmental Mutagenesis

Mutation Research 415 (1998) 167–184

Development and validation of the in vivo alkaline comet assay for detecting genomic damage in marine flatfish

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Received 27 January 1998; revised 6 May 1998; accepted 7 May 1998

breaks cannot be related to a specific exposure, when compared to e.g., DNA-adduct formation that indicates specific pollution. Nevertheless, the relative simplicity of the technique, compared to DNA-adduct determinations that require sophisticated detection techniques, makes it an interesting tool to estimate global DNA damage.

Many laboratories have put efforts in improving the usefulness of the comet assay in a wide range of research domains [1,2]. These developments resulted in a large number of different protocols of, mainly, modified methods of Singh et al. [3] and Tice [1] in a sense that "there are as many protocols as there are laboratories performing the test" (Verschaeve, 1997). Consequently, interlaboratory comparisons are compromised by the lack of standardisation.

The micronucleus test has also been used to estimate the level of exposure to contaminants by many researchers since the beginning of the eighties (for review, see Ref. [4]). This test measures structural or numerical chromosome damage and was previously most commonly used to assess genotoxicity (for review, see Refs. [5,6]). The test is a recommended indicator in environmental studies under laboratory conditions and in the field [4].

The comet assay has great potential to estimate DNA damage in fish—they are characterised by karyotypes consisting of many small chromosomes —because neither metaphases nor knowledge of the chromosome numbers are required [7]. Despite these technical advantages, the comet test has rarely been used in studies of pollution-induced DNA damage in marine fish. Most published studies have used freshwater fish such as bullheads (*Ameiurus nebulosus*) and carp (*Cyprinus carpio*; [8]), brown trout (*Salmo trutta fario*; [7]), zebra danio (*Brachydanio rerio*; [9]) and rainbow trout (*Onchorynchus mykiss*; [10]). Only Nacci et al. [11] reported on the application of the comet assay to marine flatfish (flounder, *Pleuronectes americanus*). In the marine environment, flatfish are especially suitable and recommended for monitoring purposes because these animals are sediment dwellers and sediments are known sinks for contaminants. The comet assay has mostly been applied to erythrocytes and lymphocytes in blood because these cell types can easily be sampled and cell dissociation is not needed [7], however, gills [9] and livers [10,11] have also been used.

In the present study, the usefulness of the comet assay for detecting DNA damage was tested on marine flatfish (turbot, *Psetta maximus*).

Several parameters of the comet assay were standardised, including effectiveness of tissue dissociation, cell storage, slide storage, neutralisation and fixation of the slides, intra- and interindividual variation and electrophoretic conditions. Ethyl methanesulphonate (EMS), a known genotoxic agent in vivo in fish [7], was used in the standardisation experiments. Four different tissue types were screened because genotoxic effects of contaminants are often tissue-specific [11]. The tissues were selected on procedural and/or functional criteria: (1) erythrocytes because of easy and non-destructive sampling, no further isolation steps are needed, (2) liver tissue, which is the main metabolising organ, (3) gills for their continuous contact with the water phase, and (4) kidney, which is the blood producing organ.

2. Materials and methods

2.1. Chemicals

Ethidium bromide, Giemsa, Tris(hydroxymethyl)–aminomethane, sodium hydroxide, sodium chloride and EMS were obtained from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS), RPMI 1640 medium, low melting point (LMP) and normal melting point (NMP) agarose were purchased from Gibco BRL. *N*-lauroyl sarcosine, dimethyl sulfoxide, and Triton-X 100 were obtained from Sigma (St. Louis, MO, USA), while *N*-*t*-butyl-α-phenylnitrone was obtained from Aldrich (Belgium).

Artificial seawater at a total salt concentration of 30‰ was prepared with HW sea salt (Belcopet, Belgium).

2.2. Test organisms and feeding regime

Juvenile turbot (*P. maximus*, approximately 120–200 days old, weighing between 8.27 and 47.39 g and with a length size between 8.5 and 15.0 cm were purchased from the commercial supplier France Turbot (Noirmoutier, France). The fish were kept in 500-l glass tanks containing natural seawater that was filtered through a 4-m sand bed and a 1 to 2-cm

thick layer of rinsed sand. The fish were fed on a pelleted diet, supplied by the hatchery, 2 × /day. The food quality was examined by GC-ECD and HPLC-UV/Fluorescence and considered good since the contents of organochlorines and polyaromatic hydrocarbons were below the detection limits. The water temperature was kept at 15 ± 1°C in a temperature controlled room.

2.3. Laboratory exposure system

Exposure experiments were performed in glass tanks with a working volume of 10 l artificial seawater. Randomly selected turbot were allocated into each tank (4 fish/tank). The fish were exposed to 50 mg/l ethyl EMS. Every 24 h, a 2-l volume was removed and replaced with fresh EMS-containing solution so that the final concentration added to the tanks remained 50 mg/l. This 24 h renewal was based on experience in the laboratory [12]. Gentle aeration was also provided. Samples were taken after 3 and 7 days of exposure. These sampling periods were chosen because a clear response in trout erythrocytes was seen with the comet assay after 3 and 7 days treatment with EMS [7].

2.4. Preparation of cell suspensions

Three and seven days after the primary exposure (on a total exposure period of 7 days), 2 to 3 fish/condition were sacrificed. The capacity of the electrophoresis unit (21 slides) did not allow more fish to be analysed. However, in the future more fish per experimental point could be sampled with adapted equipment. Blood was taken by cardiac puncture, and immediately afterwards the fish were killed by decapitation and dissected. No anesthetic was used. All handling of tissue dissection, dissociation, and preparations were performed on ice under yellow light, to prevent UV-induced DNA damage. The tissues were dissected and immediately washed 3 times with 9‰ NaCl solution. Different techniques for cell dissociation were tested:

- Mincing in PBS buffer + 100 mM EDTA during 20 s and 1-min periods.
- Pressing the tissue trough a 100-μm mesh filter in a syringe in the presence of PBS and 100 mM EDTA.
- Potter homogenisation in PBS containing 1 mM mercaptoethanol with a Potter–Elvehjem homogeniser.
- Incubation in 10 ml PBS containing 200 mM *N*-*t*-butyl-α-phenylnitrone (after the work of Singh [16]).
- Proteolytic digestion in RPMI 1640 medium containing 0.125% collagenase.

The cell suspensions were then centrifuged at 3020 × *g* for 10 min. After centrifugation, the supernatants were removed, and the pellets resuspended by gently tapping the culture tube. The viability of the individual cells was determined by trypan blue uptake as follows: a mixture of 500 μl, 0.4% trypan blue solution and 300 μl Hank's buffer solution were added to 200 μl of the cell suspension, mixed, and incubated for 5 min. The number of blue cells (dead) and white cells (alive) among 1000 cells were counted in a counting chamber.

2.5. Cryo-preservation of fish tissues

Three different protocols for cryo-preservation of the tissues were compared.

2.5.1. Method 1

Blood, cell suspensions, and complete organs were immersed in 500 μl RPMI containing 25% FCS and cooled, 20% DMSO was added when the temperature reached 4°C.

2.5.2. Method 2

A total of 50 μl blood was added to 500 μl RPMI containing 25% FCS and cooled, 20% glycerol was added when the temperature reached 4°C.

2.5.3. Method 3

The cells were dehydrated in RPMI containing 20% FCS and 0.25% or 0.5% sucrose for 5 min. Blood, cell suspensions and complete organs were added to 500 μl of RPMI + 20% FCS + 0.5 M sucrose + 25% DMSO.

After these preparations the cryo-tubes containing the cells were flash-frozen in liquid nitrogen.

2.6. Variance analysis

The individual variability was determined by calculating the coefficient of variation (CV = standard deviation/mean · 100).

2.7. Alkaline comet assay

The alkaline comet assay was performed under yellow light with a modified method of Tice et al. [13]. Normal slides were dipped in 1% NMP agarose gel in water and air-dried overnight. The cells (10 μl of 20-fold diluted blood samples, 10 μl for gill, 50 μl for liver and 30 μl for kidney preparations) were added to 300 μl of 0.8% LMP agarose gel, which was gently placed on top of the 1% NMP layer and covered with a coverslip. The slide was put on ice for no longer than 5 min to allow the gel to solidify. The coverslip was then removed and the slides were put in cold fresh lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% *N*-lauroylsarcosine, 1% Triton-X 100, 10% DMSO) for 1–2 h. After lysis, the discrimination power of the electrophoresis was tested under several electrophoretic conditions at 18°C (Table 4). The electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) was gently recirculated at low rate to avoid accumulation of salt near the negative pole. For the alkaline version both

Table 1
Effects of cell dissociation protocol on median of tail length (TL), tail moment (TM) and tail DNA (TDNA) after dissociation of gill, liver and kidney cells

Comet parameter	Gill median			Liver median			Kidney median		
	TL	TM	TDNA	TL	TM	TDNA	TL	TM	TDNA
Dissociation protocol (electrophoretic condition)									
1. 30 min in PBN[a]	9.5	0.50	5.31	–	–	–	10.61	0.41	4.62
2. 30 min in PBN[a]	10.06	0.52	4.82	11.45	0.41	3.97	8.94	0.42	5.11
3. 60 min in PBN[a]	9.5	0.48	5.37	8.94	0.23	2.33	9.78	0.56	5.17
4. 60 min in PBN[a]	8.94	0.31	3.11	9.5	0.52	5.59	8.38	0.25	2.62
5. 10 ml Potter–Elvehjem homogenisation in PBS + mM mercaptoethanol[b]	10.06	0.87	9.2	11.73	0.64	5.59	9.5	0.65	7.65
6. 10 ml Potter–Elvehjem homogenisation in PBS + 1 mM mercaptoethanol[c]	31.28	6.62	13.54	32.12	8.57	26.08	35.47	7.62	20.00
7. 0.5 ml Potter–Elvehjem homogenisation in PBS + 1 mM mercaptoethanol[c]	22.35	3.96	22.39	29.89	5.56	20.79	20.39	2.09	11.19
8. Pressing though 100 μm mesh filter with PBS + 100 mM EDTA[b]	15.36	1.75	12.47	29.61	5.31	18.89	18.72	1.60	8.92
9. Pressing though 100 μm mesh filter with PBS + 100 mM EDTA[c]	18.99	2.46	19.04	24.3	4.72	19.82	20.39	2.81	14.88
10. Pressing though 100 μm mesh filter with PBS + 100 mM EDTA[d]	24.58	3.96	16.72	–	–	–	14.52	1.48	11.03
11. 20 s mincing in PBS buffer with[d] 100 mM EDTA[d]	19.83	4.86	26.37	41.06	15.2	37.6	15.64	2.52	17.27
12. 1 min mincing in PBS buffer with[d] 100 mM EDTA[d]	44.69	35.96	93.02	41.62	27.96	67.92	45.25	36.37	78.51
Digestion with 0.125% collagenase in RPMI 1640 medium									
1. 30 min[b]	21.23	2.51	14.08	27.37	5.31	19.71	26.26	5.07	51.89
2. 30 min[b]	18.99	1.23	8.6	22.9	5.40	16.59	–	–	–
3. 60 min[b]	26.26	13.65	53.32	26.26	10.62	36.56	46.93	40.81	88.48
4. 60 min[b]	9.5	0.7	7.72	–	–	–	25.14	3.74	16.99
5. 90 min[b]	11.73	0.85	7.20	11.73	0.71	6.55	16.20	1.46	9.49
6. 90 min[b]	34.64	24.7	70.68	43.02	35.04	84.14	31.28	15.65	21.62
7. 90 min[b]	14.24	4.57	25.94	15.64	2.58	26.86	16.48	1.69	10.36

PBN = 10 ml PBS supplemented with 200 mM *N*-*t*-butyl-α-phenylnitrone.
The following are the electrophoretic conditions.
[a]20 min denaturation, 10 min electrophoresis (0.4 V/cm, 300 mA).
[b]20 min denaturation, 10 min electrophoresis (0.7 V/cm, 300 mA).
[c]30 min denaturation, 10 min electrophoresis (0.7 V/cm, 300 mA).
[d]40 min denaturation, 20 min electrophoresis (0.7 V/cm, 300 mA).

denaturation and electrophoresis were performed in the same alkaline electrophoresis buffer. For the 'neutral version' denaturation (20 min) was also done in the alkaline buffer, but electrophoresis was performed in neutral TBE buffer for 10 min (1 V/cm).

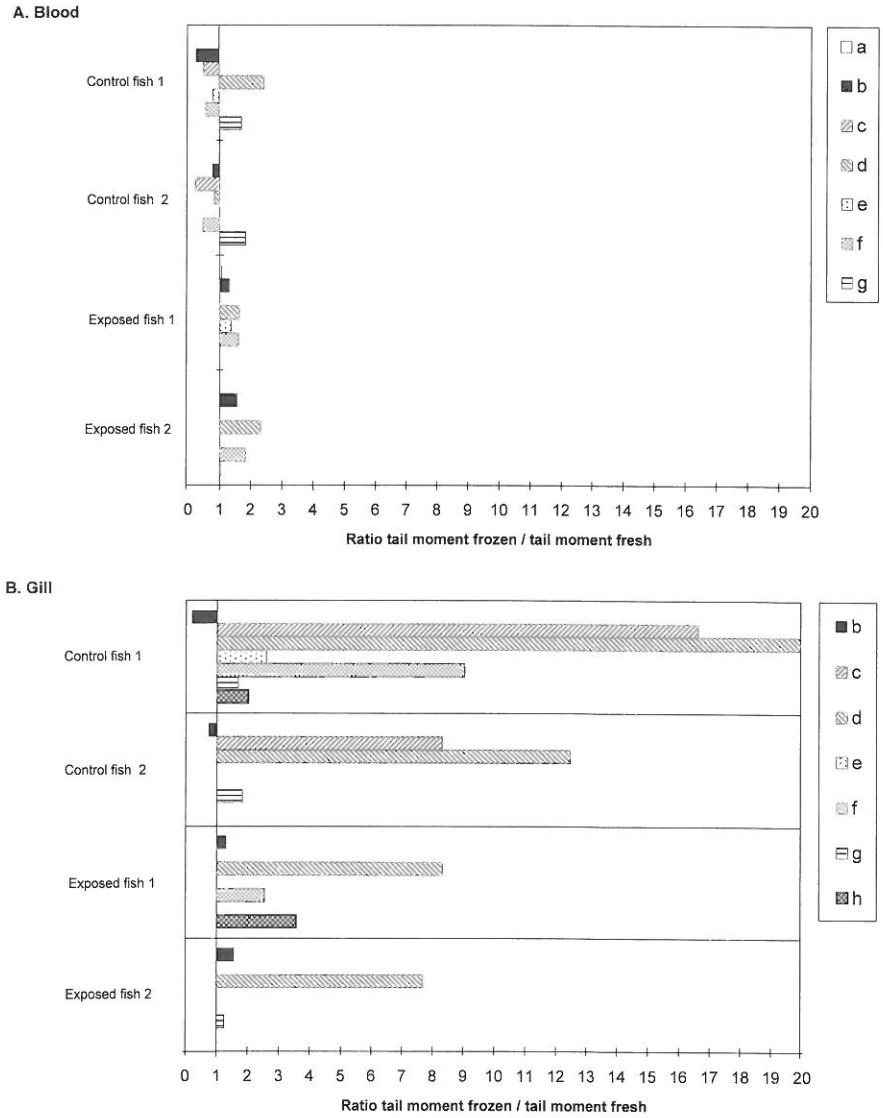


Fig. 1. Effects on the comet assay results after cryo-preservation of blood (A), gill (B), liver (C) and kidney (D) tissue in liquid nitrogen for 1 week. The results are presented as ratio 'median tail moment frozen sample/median tail moment fresh sample'. The treated fish were exposed to 50 mg EMS/l for 3 days, with an exception in protocol e where the fish were treated for 7 days. Electrophoresis conditions: 0.4 V/cm and 300 mA for 60 min. *Legend for the cryo-preservation protocols.* (a) Cell suspension in 500 μl RPMI medium supplemented with 25% FCS. After cooling of this solution to 4°C, 20% DMSO (room temperature) was added. (b) Cell suspension in 500 μl RPMI medium supplemented with 25% FCS. After cooling of this solution to 4°C, 20% glycerol (room temperature) was added. (c, d, f and g) Pre-dehydrate the blood or complete tissue for 5 min in RPMI medium + 0.25 M sucrose + 20% FCS, then for 5 min in RPMI medium + 0.5 M sucrose + 20% FCS. Flash-freeze in 500 μl RPMI + 20% FCS + 0.5 M sucrose + 3.5 M DMSO. (e) Same freezing protocol as in c (complete tissue), but fish were treated for 7 days with EMS. (h and i) Pre-dehydrate the cell suspension for 5 min in RPMI medium + 0.25 M sucrose + 20% FCS, then for 5 min in RPMI medium + 0.5 M sucrose + 20% FCS. Flash-freeze in 500 μl RPMI + 20% FCS + 0.5 M sucrose + 3.5 M DMSO. In this protocol dissociated cell suspensions were frozen instead of complete organs.

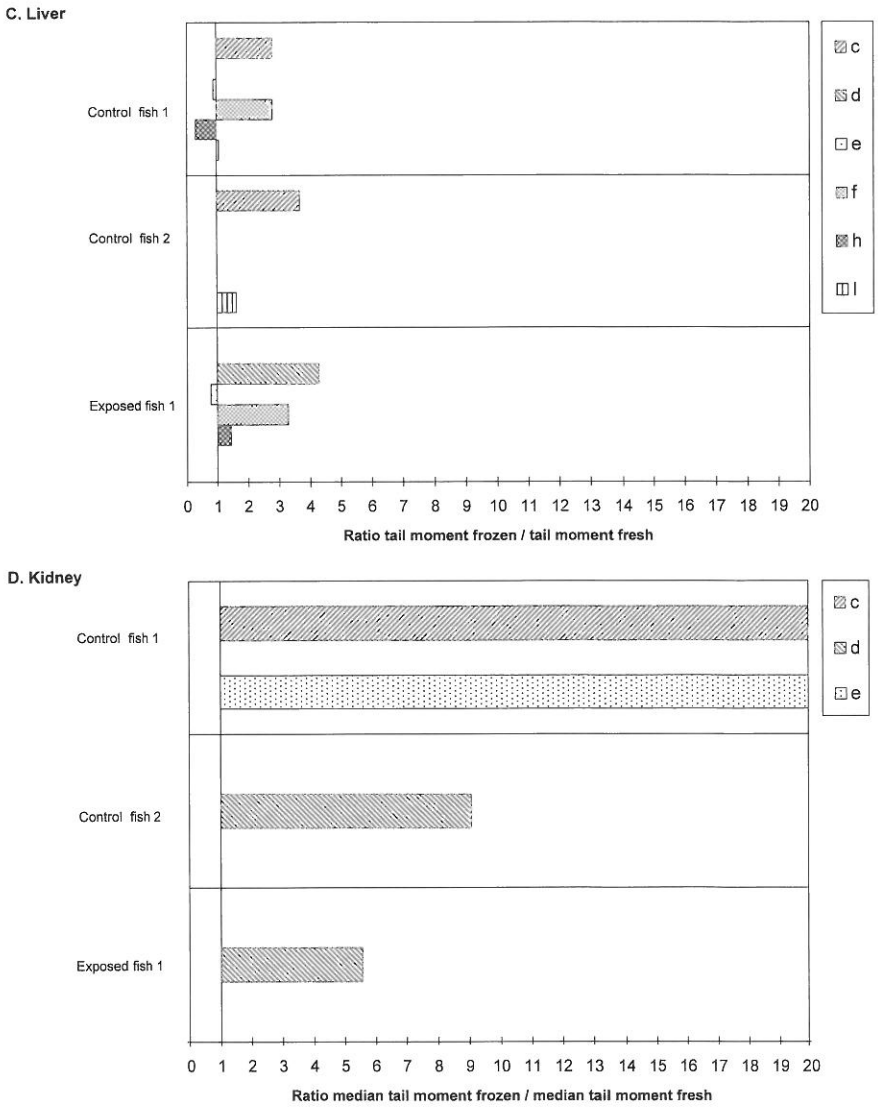


Fig. 1 (continued).

At the end of the electrophoresis, the slides were neutralised with 0.4 M Tris (pH = 7.5) and fixed in pure ethanol or methanol. Two different ways of neutralisation and fixation were tested. In *Protocol 1* the slides were neutralised 3 times for 5 min each and afterwards fixed in cold pure methanol for 5 min, as described in the work of Klaude et al. [14]. *Protocol 2* used two neutralisation steps for 15 min each and was followed by fixation in 100% cold ethanol. After 30 min, the anhydrous ethanol was replaced with 60–70% ice cold ethanol for 30 min. Finally, the slides were stored at room temperature for a maximum of 1 week, prior to the analysis. Before image analysis and processing, the slides were rehydrated with 200 μl distilled water for 10 min, then stained with 100 μl ethidium bromide (20 μg/ml) for 10 min, rinsed with distilled water and kept in a moist chamber at 4°C. The electrophoretic patterns were analysed with a fluorescence microscope (Leitz, 25 × oil immersion objective) equipped

with an image analysis system (Komet 3.0, Kinetic Imaging). A total of 50 cells/slide were measured, unless otherwise stated. All slides were coded. The parameters measured to analyse the electrophoretic patterns were: tail length (measured from the middle of the head to the end of the tail), relative DNA content in the tail, and the tail moment (tail length × relative tail DNA content). The following criteria were met to obtain precise measurements: (1) measurements near the edges of the slides were avoided;

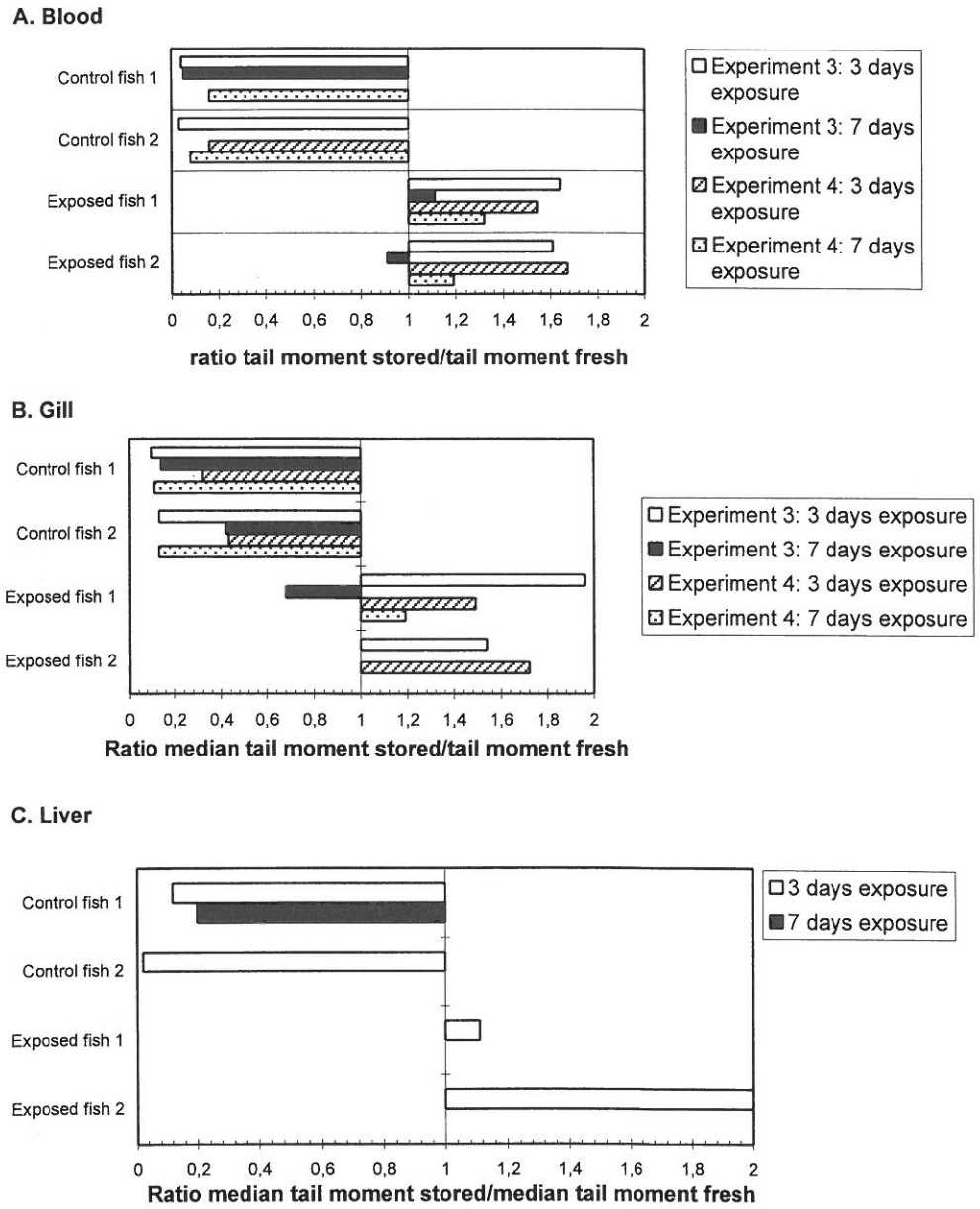


Fig. 2. Effects on comet assay results after storage of slides in lysing buffer for 1 week: blood (A), gill (B) and liver (C) tissue. The results are presented as ratio 'median tail moment stored sample/median tail moment fresh sample'. The treated fish were exposed to 50 mg EMS/l for 3 days or 7 days.

(2) no cells were scored near or in a trapped air bubble and (3) only slides with low background were used. The same operator made all measurements.

2.8. Statistics

For the comet assay, all data sets were tested for normality and homogeneity of variance and, when these criteria were met, the data were compared using Student's *t*-test. Where the data failed to meet assumptions for normality, the non-parametric Kolmogorov–Smirnov test was used. Statistical significance was set at an alpha level of 0.01 (instead of 0.05, since non-parametric tests are often too sensitive). The data of the control fish (only two control fish were used) were pooled and compared to the data of the pooled exposed fish (two or three fish/sampling time) using Student's *t*-test or Kolmogorov–Smirnov test. Pooling of two fish with the same treatment was in some cases not statistically justified, however, for statistical analysis the pooling made a comparison with the other treatments possible.

3. Results

3.1. Effects of tissue cell dissociations

The effects of different tissue cell dissociation systems were tested to optimise the isolation of individual cells from liver, gill, and kidney tissues of fish.

The results are summarised in Table 1 for the non-enzymatic digestions and for the digestion with collagenase.

Most protocols resulted in a detectable control values of tail lengths (ranging from 10.06 to 44.69 μm), tail DNA contents (ranging from 0.7% to 35.96%) and tail moments (between 7.72 and 93.02 μm) when the electrophoretic conditions were set at 0.7 V/cm and 300 mA, as described by Tice [15] (protocols 5–12 in Table 1). However, to be able to discriminate the controls from the exposed cells, it is preferable to have low control values so as to minimise a specific DNA damage in controls. This was reached by lowering the voltage of the electrophoresis (protocols 1–4 in Table 1). Dissociation in PBS in the presence of 200 mM *N*-*t*-butyl-α-phenylnitrone for 30 min or 1 h and subsequent electrophoresis at low voltage level (0.4 V/cm instead of 0.7 V/cm and 300 mA) for 10 min resulted in significant lower DNA migration in the controls. Also, the yield of single cell suspensions using this dissociation method was satisfactory. Unfortunately, a shortened electrophoresis time of 10 min did not lead to differences between controls and EMS-treated fish. Therefore, other electrophoresis conditions were applied in an attempt to make the method more sensitive (see Section 2.4). Dissociation using collagenase did not result in lower control values than the dissociation with 200 mM *N*-*t*-butyl-α-phenylnitrone.

In all further experiments the dissociation of gill, liver, and kidney tissues were done in the presence of *N*-*t*-butyl-α-phenylnitrone. The viability of the cells, measured with trypan blue exclusion, was in all cases high ($> 94\%$ for liver and kidney cells and $> 72\%$ for gill cells).

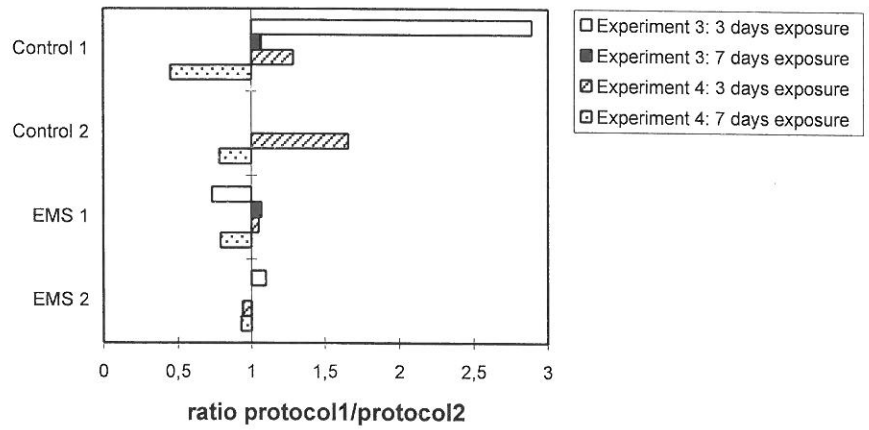
3.2. Effects of cryo-preservation of fish tissues and cell isolates on comet parameters

Cell samples were prepared by cryo-preservation in the presence of a permeating agent (DMSO or glycerol) or in the combined presence of a permeating and non-permeating agent (sucrose). The effects of the preservation protocol with cells isolated from control and exposed animals were investigated.

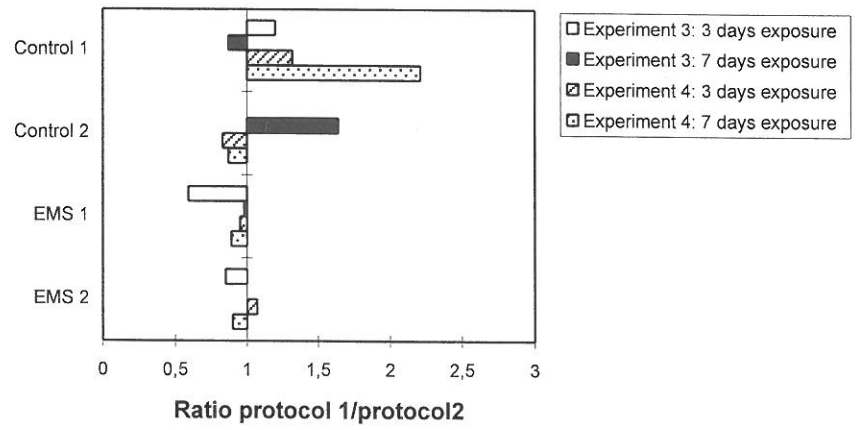
All results (Fig. 1A–D) were expressed as the ratio 'median tail moment in the cryo-preserved sample/median tail moment in the fresh sample'. The ratio clearly demonstrates the storage effects. A ratio lower or higher than 1 indicates that the tail

Fig. 3. Effects of fixation and neutralisation protocols in the comet assay: blood (A), gill (B) and liver (C) tissue. Two protocols for neutralisation and fixation of the slides are compared. Protocol 1 = neutralisation 3 times for 5 min with 0.4 M Tris, and fixation in 100% cold ethanol for 5 min (after the work of Klaude et al. [14]). Protocol 2 = neutralisation 2 times for 15 min and fixation in 100% cold ethanol for 30 min, followed by 60–70% cold ethanol for 30 min. Results are presented as 'ratio protocol 1/protocol 2'. Both protocols were compared twice.

A. Blood



B. Gill



C. Liver

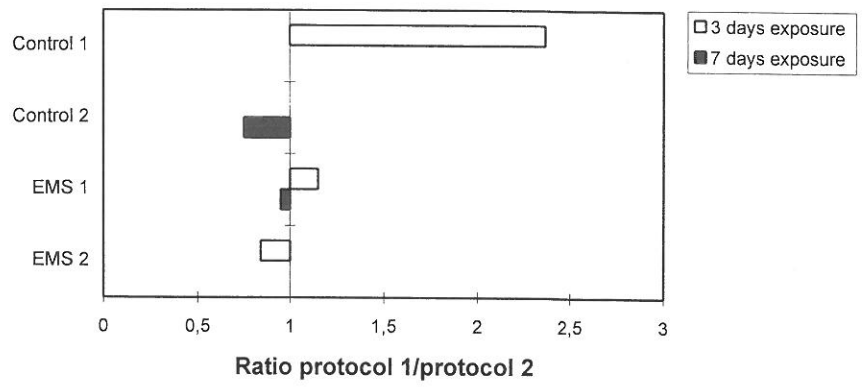


Table 2
Range for the coefficient of variation for individual fish (CV = SD/mean · 100)*

Tissue	Range for tail length	Range for tail moment	Range for tail DNA
Blood controls	7.34%–110.0%	25.9%–106.8%	19.5%–88.6%
Blood exposed	4.8%–55.0%	8.6%–111.9%	5.0%–89.2%
Gill controls	5%–38.5%	10.3%–14.4%	8.9%–107.4%
Gill exposed	6.8%–15.9%	8.8%–100%	4.1%–87.6%
Liver controls	7% –60%	17.6%–95.2%	14.1%–93.6%
Liver exposed	5.5%–17.2%	8.7%–95.5%	4.8%–88.2%
Kidney controls	3.03%–32.0%	21.6%–106.3%	17.1%–99.3%
Kidney exposed	4.88%–19.0%	8.77%–101.3%	6.0%–87.8%

*This reflects the intraindividual variation within fish (50 cells were measured on one slide).

moment has, respectively, decreased or increased after storage. For 'protocol a' (Fig. 1A; RPMI + 25% FCS + 20% DMSO) a ratio of 1.06 was calculated for the ratio of the tail moments, indicating only a slight increase in DNA damage. The other freezing techniques ('RPMI 1640 medium + 25% FCS + 20% glycerol' and 'RPMI 1640 medium + 25% FCS + 0.5 M sucrose + 25% DMSO') revealed 'frozen/fresh' ratios higher than 1 in most cases. For gill (Fig. 1B) and kidney (Fig. 1D) the ratios were sometimes as high as 20. In some cases (e.g., Fig. 1A and B protocol b, Fig. 1C protocol h) comet migration was lower after freezing (ratio of frozen/fresh is < 1). This was generally the case in control fish. None of the tested protocols provided a perfect preservation of the fish tissues.

3.3. Effects of storage of slides in lysing buffer

Effects of prolonged storage in lysing buffer on the integrity of DNA damage of the different fish tissue nuclei were assessed. The results, expressed as 'ratio median tail moment stored sample/median tail moment fresh sample', are presented in Fig. 2A–C. Again, a ratio higher than 1 indicates that the number of DNA breaks have increased after storage in the

Table 3
Interindividual variation between two animals from the same treatment

Exposure	Experiment no.	Blood CV			Liver CV			Gill CV		
		TL	TM	TDNA	TL	TM	TDNA	TL	TM	TDNA
Controls 3 days	1	0.18	23.55	1.45	–	–	–	–	–	–
	2	39.96	48.89	24.63	–	–	–	17.48	43.18	19.09
	3	11.42	8.62	1.76	39.84	47.06	14.07	–	–	–
	4	0.18	2.34	0.80	–	–	–	1.34	29.10	24.85
Controls 7 days	1	10.09	12.74	0.22	15.46	26.40	18.28	24.70	51.77	32.50
	2	–	–	–	–	–	–	–	–	–
	3	–	–	–	4.51	13.85	12.54	22.31	28.46	14.86
	4	22.04	37.13	18.62	–	–	–	0.40	2.68	2.70
Exposed 3 days	1	2.31	12.86	12.51	–	–	–	–	–	–
	2	6.33	12.34	2.05	–	–	–	0.58	1.82	2.38
	3	0.13	1.18	1.40	17.91	38.21	21.13	0.55	15.26	16.13
	4	0.31	6.68	3.57	–	–	–	3.82	2.78	0.67
Exposed 7 days	1	5.6	6.38	3.33	4.00	13.89	10.28	–	–	–
	2	–	–	–	–	–	–	–	–	–
	3	15.94	15.91	0.06	6.45	5.56	1.00	61.92	8.13	6.46
	4	0.96	1.16	1.56	–	–	–	6.95	14.46	7.70

The exposed fish were treated with 50 mg EMS/l for 3 or 7 days.
The CV was calculated as the SD/mean · 100.

lysing buffer, a ratio lower than 1 indicates a decrease.

Slides from control fish showed a clear decrease (Fig. 2A) in tail moments after 7 days of storage as compared to fresh slides. On the other hand, the tail moments in slides of EMS treated fish, exposed to 50 mg EMS/l during 3 to 7 days, generally slightly increased when stored in lysing buffer (highest ratio: 2). In two cases decreases in tail moments were observed in blood (Fig. 2A) and gill (Fig. 2B) slides from 7 days exposed animals in experiment 3. These results indicate the that storage of slides in lysing buffer for only 7 days may produce changed comet patterns. Since no other storage techniques are cur-

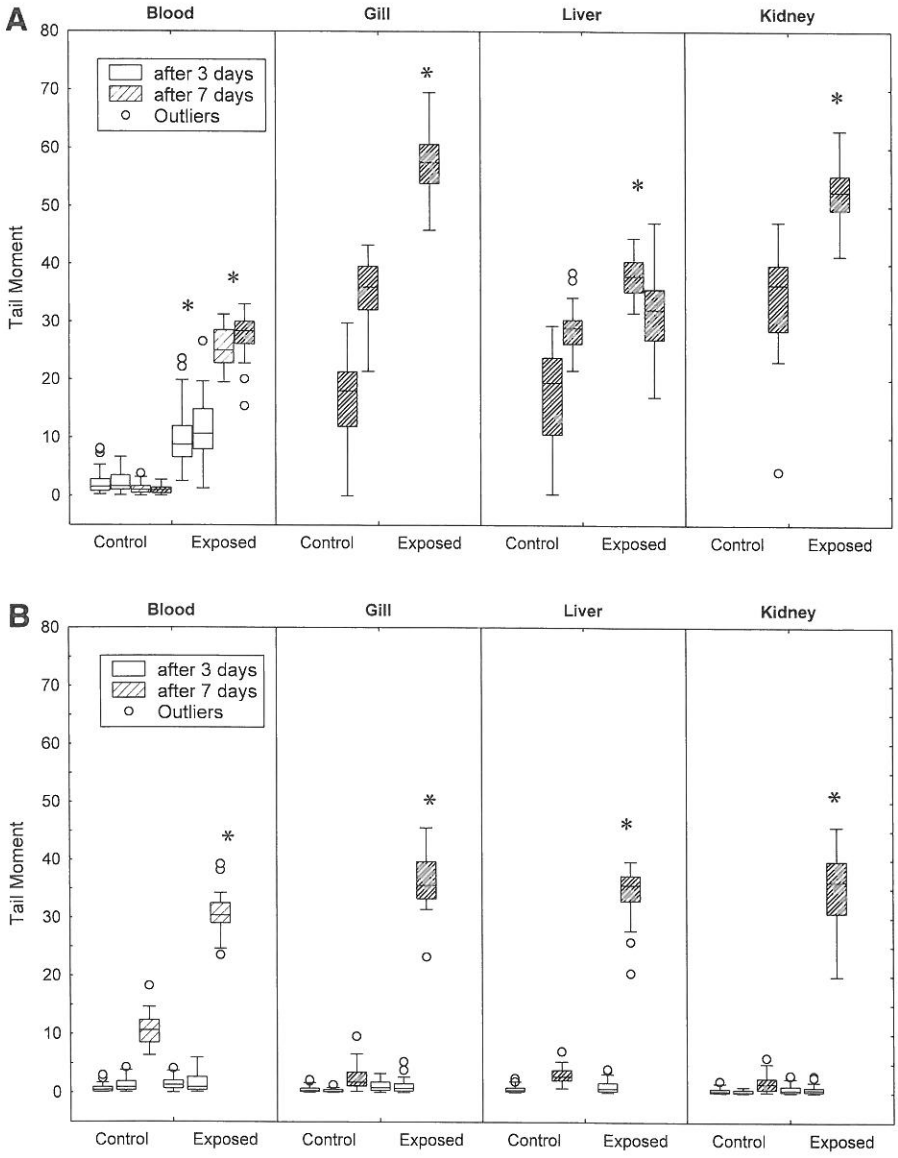


Fig. 4. Effects of EMS exposure in four replicate experiments (A–D): box-plots for tail moment after 3 or 7 days exposure to 50 mg EMS/l. Each box represents an animal, and outlier value refer to cells. The edges of the boxes represent the 25% and 75%. The wiskers show the non-outlier range. Outlier = if data point > UBV + 1.5 · (UBV − LBV) or data point < LBV − 1.5 · (UBV − LBV), where UBV = upper value of the box and LBV = lower value of the box. Electrophoretic conditions: 20 min denaturation, 60 min electrophoresis (0.4 V/cm, 300 mA). * = Exposed vs. control: $p < 0.001$.

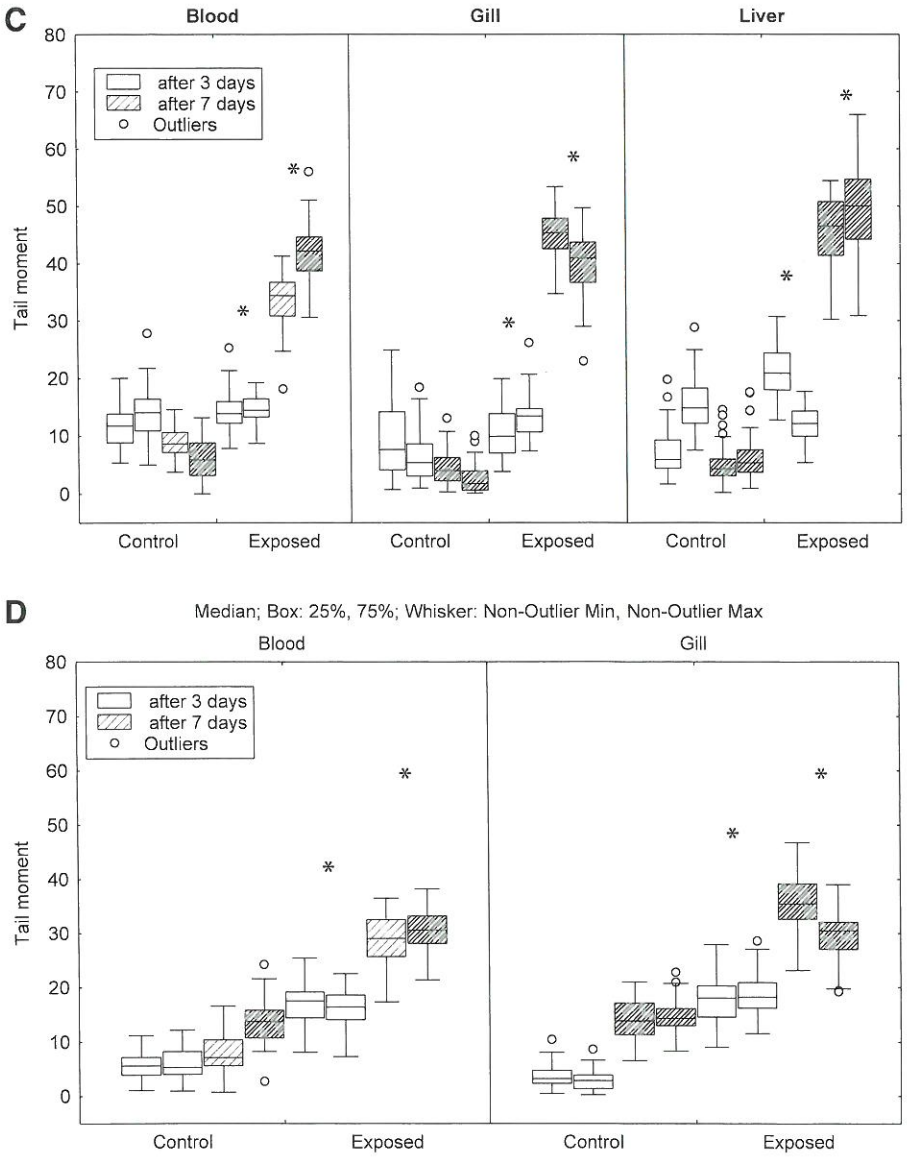


Fig. 4 (continued).

rently available, this type of preservation is considered as an acceptable alternative to freezing of tissue samples.

3.4. Effects of neutralisation and fixation of slides

The influences of two neutralisation and fixation protocols, according to Klaude et al. [14] (protocol 1) and Singh [16] (protocol 2) were examined and compared. The slides for the two fixation methods were run at the same time.

The results are expressed as the ratio 'median tail moment protocol 1/median tail moment protocol 2' (Fig. 3A–C). If protocol 1 would lead to an improved discrimination between the control and exposed measurements the ratio for the control fish are expected to be always lower than 1 and/or the ratio for the exposed fish would be always higher than 1.

Since none of the protocols led to an improved discrimination between controls and exposed fish, protocol 1 was chosen as the standard neutralisation and fixation procedure in all experiments because it is less time-consuming.

3.5. Intrasample and interindividual variation of the comet migration

The intraindividual and interindividual (between individuals) variation of the comet migration was examined. The CV for one slide was calculated as: CV = SD/mean · 100. The range for the CV of the different comet parameters for the blood, liver, gill and kidney is given in Table 2. Apart from some exceptions (6%) the ascending order was: CV of TM > CV of tail DNA > CV of TL in 81%, and CV of TM > CV of TL > CV of tail DNA in 13% of the cases. All tissues show both extremely high and low CVs, which indicates that high or low CVs are not intrinsically bound to a certain tissue type. The CV of controls is most often higher than that of exposed animals. Besides intraindividual, also interindividual variability can be important in the interpretation of comet results, i.e., variability between two fish treated in the same way. This variability is also expressed as the coefficient of variation (CV = SD/mean · 100). The mean and SD of different comet parameters from fish treated similarly were used to calculate the CV for control and exposed fish in experiments 1 to 4 (Table 3). For this interindividual variation the CV for TM (ranging from 2.34% to 51.77% for controls, and from 1.16% to 38.21% for exposed fish) was in 64.7% of the cases higher than the CV for TL (ranging from 0.18% to 39.96% for controls and from 0.13% to 61.92% for exposed) and tail DNA (ranging from 0.22% to 32.50% for controls and from 0.06% to 21.13% for exposed fish). In most of the cases, the CV between control fish is higher than the CV between exposed fish.

3.6. Tissue specific responses of EMS-induced DNA damage

Tissue specific responses of blood, liver, gill and kidney were studied in fish exposed to 50 mg EMS/l for 3 or 7 days. In experiment 1 the exposure time was prolonged until the 14th day. The cells isolated from all exposed tissues showed increased tail lengths and tail DNA at day 3 during the exposure (Fig. 4A–D) and the effects had further increased in fish exposed for 7 days. The significance levels are shown in Fig. 4.

3.7. Effects of denaturation and electrophoresis

An attempt to improve the sensitivity of the test was made by changing the denaturation and electrophoresis times. Four combinations of varying denaturation and electrophoresis conditions in alkaline

Table 4
Effects of electrophoresis conditions on comet assay results: blood, gill, liver and kidney

Electrophoretic condition		Blood		Liver		Gill		Kidney	
		Mean TM	Median TM	Mean TM	Median TM	Mean TM	Median TM	Mean TM	Median TM
Condition A	Controls	1.20 ± 1.16	0.89	0.50 ± 0.64	0.27	0.47 ± 0.74	0.32	0.34 ± 0.46	0.20
	Exposed	8.63 ± 5.82	7.47	8.32 ± 3.13	8.54	7.57 ± 3.37	7.52	7.66 ± 4.14	7.06
Condition B	Controls	2.54 ± 1.60	2.16	2.14 ± 2.26	1.60	1.21 ± 2.51	0.67	1.00 ± 1.33	0.71
	Exposed	17.55 ± 4.47	17.68	11.45 ± 5.02	11.24	14.72 ± 7.24	13.63	11.41 ± 8.07	9.96
Condition C[a]	Controls	3.97 ± 2.01	3.74	1.74 ± 1.42	1.31	1.33 ± 1.33	1.11	0.53 ± 0.54	0.30
	Exposed	15.62 ± 2.31	15.45	18.79 ± 2.54	18.93	14.55 ± 2.65	13.61	17.39 ± 3.92	17.01
Condition D[a]	Controls	10.61 ± 2.75	10.65	3.02 ± 1.50	2.71	2.57 ± 2.17	1.74	2.05 ± 2.18	1.54
	Exposed	30.60 ± 3.72	30.33	34.12 ± 4.54	35.58	35.32 ± 5.16	35.49	34.63 ± 6.94	36.16

Data from two fish were pooled for each condition.

[a]A total of 50 cells/slide were measured in conditions A and B, 25 cells/slide in conditions C and D.

Legend for electrophoretic conditions: Condition A: 20 min denaturation, 10 min electrophoresis (0.4 V/cm, 300 mA). Condition B: 20 min denaturation, 10 min electrophoresis (0.7 V/cm, 300 mA). Condition C: 40 min denaturation, 20 min electrophoresis (0.4 V/cm, 300 mA). Condition D: 20 min denaturation, 60 min electrophoresis (0.4 V/cm, 300 mA).

Table 5
Mean tail DNA ± SD, expressed as percentage of total DNA, of tissues of fish treated with 50 mg EMS/l

Tissue	0.4 V/cm (60 min), condition D	0.7 V/cm (10 min), condition B
Blood	75.27 ± 6.16	56.47 ± 11.88
Liver	80.58 ± 5.91	10.73 ± 9.42
Gill	78.53 ± 12.13	46.37 ± 18.42
Kidney	76.02 ± 10.21	36.46 ± 20.39

buffer (300 mM NaOH, 1 mM EDTA, pH > 13) were tested (Table 4).

The results of this approach are shown in Table 4. Small but significant ($p < 0.001$) differences between the DNA patterns of exposed and control animals were obtained after electrophoresis at 0.4 V/cm during 10 min (Table 4, condition A). A higher voltage (0.7 V/cm) resulted in a better separation between controls and exposed fish, but the tail variability within the pools of exposed cells became higher (see Table 5). The best separations between control and exposed sets were obtained by prolonged electrophoresis time up to 60 min at low voltage (0.4 V/cm), as specified in condition D. This electrophoresis condition was used in all further experiments.

Replacement of the alkaline buffer with a neutral TBE buffer in the electrophoresis step but not in the denaturation step resulted in less clear discrimination in tail moments between exposed and control fish. The protocol and electrophoretic conditions for the neutral version followed the method described by Koppen [17] for plant cells. The results are shown in Table 6.

4. Discussion

There is a clear need for sensitive methods that are suitable to assess the chronic impact of contaminants on organisms. In 1997, the ICES ACME concluded that the comet assay is a promising technique for the detection of DNA damage but also that more research is needed to get the technique ready for future inclusion into an integrated biological/chemical marine environmental monitoring strategy [18]. Klaude et al. [14] argued that to achieve this goal the research should focus on the influences of natural and artificial factors and on the provision of a fully standardised protocol. This report seeks to standardise the comet assay procedure and investigates the effects of cell dissociation techniques, tissue cryopreservation, slide storage, electrophoretic conditions, neutralisation and fixation.

One of the advantages of the comet assay is that it provides information at the single cell level. This, however, implies that a suspension of well preserved single cells is needed. Many options are open and some were already dealt with in earlier publications [9,19]. The use of the comet assay for monitoring purposes requires quick, simple, and inexpensive dissociation techniques. With regard to tissue dissociation, sufficient cells and an acceptable low DNA damage level in control cells were obtained by incu-

Table 6
Values for tail DNA (mean ± SD) after alkaline and neutral electrophoresis

Tissue	Fish	Alkaline electrophoresis (after 3 days)	Neutral electrophoresis (after 3 days)	Alkaline electrophoresis (after 7 days)	Neutral electrophoresis (after 7 days)
Blood	Control 1	22.34 ± 4.00	7.37 ± 4.43	36.97 ± 12.72	–
	Control 2	22.99 ± 4.03	6.82 ± 4.92	48.19 ± 9.45	6.55 ± 4.41
	Exposed 1	32.31 ± 2.70	8.53 ± 5.88	73.07 ± 8.16	8.75 ± 7.17
	Exposed 2	32.45 ± 2.29	8.39 ± 5.47	74.71 ± 6.52	10.34 ± 6.41
Gill	Control 1	20.16 ± 12.49	6.80 ± 4.30	46.89 ± 9.20	8.86 ± 4.58
	Control 2	14.13 ± 7.87	8.80 ± 5.71	48.72 ± 7.94	5.54 ± 5.21
	Exposed 1	55.54 ± 9.36	11.29 ± 7.90	81.74 ± 7.08	18.63 ± 14.56
	Exposed 2	55.01 ± 8.70	9.50 ± 6.19	73.30 ± 7.56	11.53 ± 6.65

Both electrophoresis steps were preceded by 20 min denaturation in a mixture of 300 mM NaOH and 1 mM EDTA. For the alkaline protocol electrophoresis was performed in the same buffer for 60 min (0.4 V/cm).
The neutral electrophoresis was performed in TBE buffer for 10 min (1 V/cm).
Fish were exposed to 50 mg EMS/l for 3 or 7 days.

bating the examined tissues (liver, kidney and gill) in a 200-mM *N-t*-butyl-α-phenylnitrone solution in PBS for 1 h. This cell suspension was then filtered and centrifuged. Further dissociation by means of mincing or proteolytic digestion was redundant. Nacci et al. [11] described another simple technique in the absence of enzymes, but the disadvantage is that it is too time-consuming, especially when many samples should be processed, as for pollution assessment.

ICES ACME recommended the handling of a total of 25 fish/sampling site [20]. This may rapidly lead to a chaotic workload especially when several subsamples are needed per individual. The use of the comet assay in aquatic field surveys usually also involves many samples. An effective method for the preservation of samples is thus clearly a prerequisite to broaden the application of this technique. Two preservation techniques, namely, storage in lysing buffer and cryo-preservation were examined. Nacci et al. [11] stored slides in lysing buffer for periods of up to 4 months. However, to our knowledge no reports on effects of storage in lysing solution exist. Comet analysis of slides from blood, gill, and liver tissues which were stored in lysing buffer for 1 week (Fig. 2) revealed that the number of DNA breaks decreased significantly in controls (tail moment ratios lower than 1, ranging from 0.02 to 0.43), whereas slight increases were noted in the EMS exposed samples (ratios higher than 1, raging between 1.11 and 2.0) as compared to slides for which the comet protocol proceeded after 1 h of lysis. Consequently, the changes that occur after storage in lysing buffer result in higher but specific signals that may be erroneously interpreted as increased levels of DNA damage. It is also difficult to clarify these contradicting changes but it may be expected that diffusion of very small DNA fragments are likely to occur in controls, which can result in a higher background and thus a final lower DNA content of the comet tail. It should also be noted that DNA damage may be caused by oxidation during storage. Adding DMSO to the lysing buffer can prevent oxidative damage [13]. A possible explanation for the increase in tails in exposed cells is that the prolonged storage at pH 10 may also induce the conversion of alkali labile sites to DNA strand breaks.

Freezing of isolated cells and tissues as well as whole organisms at -196°C led in the majority of the cases to an increase in DNA damage which was not proportional between fresh and cryo-preserved exposed and control samples. In other words, the tail moments of control and exposed samples had not equally increased after cryo-preservation. Fig. 1 clearly shows that many ratios are higher than 1.

An overall conclusion is that the examined techniques for cryo-preservation with permeating (DMSO or glycerol) and non-permeating (sucrose) agents did not satisfactorily contribute to stable long-term storage of biological samples. However, the changes in the comet patterns remain lowest in blood, but highest in gill and kidney. The former tissue might be a good candidate for freezing, after perfection of or changes to the storage protocols. Still comet data, obtained from preserved samples, should therefore be treated with caution. The potential of cryo-preservation of tissues in liquid nitrogen is recognised and the actual research currently focuses on the long-term preservation of living embryos and bone marrow cells [21]. It may be expected that these developments contribute to better procedures to preserve the integrity of the samples for comet analysis. At present other protocols using trehalose and slow freezing are being evaluated.

The use of dried slides for sample preservation pending image analysis was first introduced by Klaude et al. [14]. The use of dried slides facilitates transport and the slides can be rehydrated at any suitable time. The protocol consists of three neutralisation steps for 5 min each and a fixation step in cold absolute ethanol or methanol during 5 min. However, Singh [16] emphasised that better results may be obtained with prolonged neutralisation and fixation. If so, one would expect the ratios for controls in Fig. 3 to increase (i.e., the fixation according to Singh would give lower control values) and/or the ratios for exposed to decrease (i.e., the fixation according to Singh would result in longer tails), which should lead to a better discrimination between control and exposed animals. This was not always the case. Therefore, we can conclude that Singh's technique did not enhance the detection of DNA damage nor provide a better discrimination between control and exposed fish compared to the protocol suggested by Klaude et al. [14]. Neither of the two techniques is clearly superior to the other. The latter technique was applied in all experiments because it

is less time consuming. The results indicate, however, that neutralisation and fixation can influence the outcome of an experiment. It is therefore suggested that the operational conditions of these intermediate steps should be described in detail.

Intraindividual heterogeneity in response and variability among similarly treated animals, were examined in all exposure experiments by comparing the coefficients of variation (CV = SD/mean · 100). For the intraindividual variation (Table 2) these calculations revealed that: (i) the CV for TM is higher than for tail DNA, both are higher than the CV for TL, with few exceptions; (ii) an extremely low or high CV is not intrinsically characteristic for a certain tissue type; (iii) the variability within one slide from control fish is higher than in slides from exposed fish. When interindividual variation is considered the variation between control fish is often higher than the variation between exposed fish (Table 3). This is particularly so for the tail moment. On the contrary, De Boeck et al. [22] recently found that variability between human donors could only be observed after treatment and not in control cultures. As a general conclusion, it can be argued that the variation within one slide (intraindividual) is often higher than the interindividual variation (between individuals).

Four replicate experiments, in which fish were exposed to 50 mg EMS/l for 3 or 7 days, were conducted. In this way we could determine the sensitivity of flatfish and tissue specific responses to a direct acting mutagen and check whether all tissues are equally sensitive to this mutagen. A high interexperimental variation was observed (i.e., the replicate experiments have a low reproducibility). Nevertheless, the different tissues show similar response patterns in all experiments. The use of *tail length* as discriminating parameter did not provide consistent evidence for genotoxic effects of EMS. A clearer tissue responsiveness was observed when the *tail DNA* of control and exposed animals was compared. Especially for *tail DNA*, clear differences in responses to EMS exposure were found for the time course of the exposure depending on the tissue type. Gill cells seemed more sensitive than blood cells and the responsiveness of liver cells was even lower. For kidney cells no clear trend was seen. A similar tissue-dependent response was noted by Sasaki et al. [19]. In our experiments exposed blood cells showed significantly higher tail lengths, tail moments, and tail DNA after 3 days (except in replicate 2) and even higher tail moment and tail DNA after 7 days, while significant effects on liver tissue were only observed at day 7. Gill tissue gave the most uniform results: all three parameters increased significantly upon exposure at both sampling moments. All parameters in kidney tissue also increased except tail DNA at day 3 of exposure. These results clearly indicate that the assessment of genotoxic effects of contaminants can best be achieved in tissues such as blood or gills based on tail DNA rather than tail length. The responses are time-dependent. In Fig. 4 only 'tail moment' is presented. Tail moment takes into account both tail length and tail DNA, and is therefore often a valuable parameter to use.

Our findings on time response in fish related to cytogenetic effects of contaminants using the comet assay confirm the findings of several authors. Deventer [9] also saw a clear time-related genotoxic effect in liver and gill cells from fish exposed to 80 μM MMS during 8 h. Nacci et al. [11] observed a significant increase in comet tails of flounder erythrocytes exposed to hydrogen peroxide concentrations between 1 and 100 μM. After exposure of flatfish to B[*a*]P-spiked sediment, only a significant effect was seen in gut, but not in blood and liver. The absence of responses in the liver may be sought in the production of diverse nuclear lengths and qualities from crude liver homogenates which may obscure the detection of effects of exposure on specific cell types. Multiple washing of the tissues, as shown in this paper, can prevent contamination of specific tissue slides with erythrocytes. Pandrangi et al. [8] demonstrated increased DNA damage after exposure of bullhead (*A. nebulosus*) and carp (*C. carpio*) to cyclophosphamide. Devaux et al. [10] also measured increased tail lengths with the comet after in vitro exposures of hepatocytes to B[*a*]P and hydrogen peroxide.

All of these previous publications, including this one, prove that the comet assay is indeed able to detect DNA damage caused, by different classes of mutagens in fish. The response will, of course depend on the experimental conditions, the species, the cell type, the mutagen, and the duration of exposure.

There is no standardised protocol for the comet assay at the moment. Lysing conditions, denatura-

tion, electrophoresis times and electrophoretic conditions vary considerably between different published reports and laboratories. Apparently different cell types require different unwinding times, current, buffers etc. [14,23,24]. This resulted in a wide variety of different protocols. In this paper, an attempt was therefore made to establish an uniform and reproducible comet assay protocol suitable for use with different tissue types of fish. Our studies on the effects of the electrophoretic conditions were mainly based on the recommendations of Singh [16] to perform a long-term low voltage potential electrophoresis at 0.4 V/cm instead of shorterterm at higher voltage (usually 0.7 V/cm). Low voltage electrophoresis seems to give a better discriminating power. The choice of the electrophoretic conditions should thus ideally be adapted to the latter condition and minimise the aspecific DNA damage in controls [23,24]. Optimal comet conditions may be different for different organs [19]. The tissue-specificity of the electrophoretic resolution was clearly shown by Nacci et al. [11]. The optimal conditions to demonstrate DNA damage in flounder (*P. americanus*) cells are 15 min of unwinding followed by 20 min electrophoresis, while oyster (*Crassostrea virginica*) haemocytes needed a longer unwinding period (up to 60 min) and 5 min electrophoresis. Pandrangi et al. [8] successfully used 15 min unwinding in an alkaline buffer and 20 min electrophoresis (25 V, 265–270 mA) as a standard protocol for blood cells of bullheads (*A. nebulosus*) and carp (*C. carpio*). The authors also revealed that prolonged running increased the tail length. Influences of electrophoresis duration were also mentioned by Navarrete et al. [23]: the difference in migration after 2 and 4 Gy of γ-irradiation was only clear when electrophoresis took place for 20 min, but not after only 10 min.

Our conclusions are that electrophoresis at low voltage (0.4 V/cm) for 60 min, instead of short term (10 min) electrophoresis at higher voltage (0.7 V/cm) led to a better discrimination between control and exposed fish. One disadvantage associated with prolonged electrophoresis may be the possible occurrence of additional DNA damage due to free radicals generated in the alkaline electrophoresis solution [16]. Therefore, it is suggested that antioxidants should be added to the electrophoresis solution for a better detection of contaminant-induced DNA damage in exposed cells. The presence of radical scavengers in the electrophoretic solution and the use of an intense fluorescent dye, YOYO-1, would further enhance the sensitivity, according to Singh [16].

An overall conclusion is that the comet assay may be an interesting monitoring tool to demonstrate genotoxic exposures and investigate the health impacts of DNA damage, repair, and recovery in species of environmental concern [11]. In this respect, three main advantages were identified: (i) any nuclear cell type can be used; (ii) only small samples are needed (5000–50 000 cells); (iii) the assay is quick, sensitive and inexpensive. The comet assay may be used to replace or complement measurements of chromosomal aberrations, micronucleus, and sister chromatid exchanges. The sensitivity and specificity of the assay can be improved by the use of purified repair enzymes applied to the DNA during the course of the comet assay procedure [2]. Some important disadvantages remain: (i) the inability to detect genetic damage such as, e.g., gene mutation; (ii) the generated DNA lesions are not fixed and can be repaired so that the estimation of long-term effects on the organism is difficult; (iii) high inter- and intraindividual variability obscures clear interpretation of the results; (iv) quantification of the number of breaks has not been possible. In this study, statistically significant results were obtained for all tissues with the comet assay. Evidence was obtained that for fish tissues, the following conditions are optimal: solid tissue dissociation in PBS + 200 mM *N-t*-butyl-α-phenylnitrone, electrophoresis at 0.4 V/cm for 60 min, fixation and neutralisation according to Klaude et al. [14] and tissue or slide storage should be prevented or restricted to short periods. At the present time, the results of an in situ biomonitoring field study using the comet and micronucleus test to detect genotoxic effects in fish blood, liver, gill, and kidney tissues are being evaluated. This will contribute to the future applicability of these tests for monitoring purposes.

Acknowledgements

The authors are very grateful to Omer Decock, Marie-Jeanne Vandenbouhede and Maguerite Van-Mechelen for their expert technical assistance. Kathy

Belpaeme is a Research Assistant of the Fund for Scientific Research-Flanders (FWO). This research was funded by the Fund for Scientific Research-Flanders and the Agricultural Research Centre (Sea Fisheries Department).

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 ISSN/1383-5718/1998/$19.00

Publication information: *Mutation Research, Genetic Toxicology and Environmental Mutagenesis* (ISSN 1383-5718). For 1998 volumes 396–420 are scheduled for publication. Subscription prices are available upon request from the Publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where Air delivery via SAL mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available upon request. Claims for missing issues should be made within six months of our publication (mailing) date.

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