The single cell gel electrophoresis technique or comet test for monitoring dioxin pollution and effects.

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INTRODUCTION

The Single Cell Gel Electrophoresis (SCGE) technique or comet test was introduced in 1988 as a new, rapid and inexpensive method for detecting DNA single strand breaks and alkali labile sites[1]. Although not yet fully validated, the method may be considered an important tool for biomonitoring and genetic toxicity testing. Taking into account its likely applicability to any eukaryotic organism and cell type, we have adapted the SCGE analysis to earthworm coelomocytes. When target organisms such as earthworms are exposed to a contaminated environment, the SCGE technique will measure the cumulative DNA damage caused by all (geno)toxic pollutants that are available to the organism. Though differences in sensitivity towards various classes of pollutants, or towards individual compounds within a class, may well exist, the method clearly is not a specific assay for a particular pollutant or class of pollutants, e.g. dioxins.

In this paper we report some experiments aimed at estimating the response towards dioxins, in order to find out whether an optimized earthworm SCGE assay nevertheless might be a promising tool for monitoring the bioavailability and (geno)toxic effects of dioxins in terrestrial environments. In such case, adaptation of the SCGE technique for the analysis of other organisms might easily broaden its scope to other environmental compartments.

THE EARTHWORM SINGLE CELL GEL ELECTROPHORESIS TECHNIQUE

In the SCGE assay a cell suspension is brought into a lysis buffer enabling the release of DNA from individual cells. Then the DNA is successively subjected to alkali treatment for unwinding (or denaturation) and electrophoresis, which will cause DNA fragments to migrate according to their size and molecular weight. If substantial DNA damage has occured a comet-like figure will form; the longer the tail length, the more the DNA damage. After staining with ethidium bromide, slides are analyzed using a fluorescence microscope to measure DNA tail lengths.

The comet tests presented in this paper were performed on adult earthworms (Lumbricus terrestris, ssp. castaneus; approximative weight 1.25 g). All earthworms, collected from an unpolluted site, were allowed to acclimate to laboratory conditions for several weeks before the tests. According to preliminary investigations we obtain reproducible results with such animals.

TWO EXAMPLES OF EARTHWORM SCGE-TESTS IN RELATION TO DIOXINS

a. Exposure of earthworms to soils spiked with a mixture of the 'dirty seventeen' PCDD/PCDF congeners

For this experiment a dioxin standard solution in nonane was used, containing the 17 most toxic PCDD/PCDF congeners at about the same concentration, with exception of the octachlorinated congeners which were present at a level twice as high. From this solution appropriate dilutions were prepared, using hexane, and added to standardized dry soil samples. After evaporation of hexane (2 days, 37 °C) the soils were humidified with 65% phosphate buffer saline (PBS; pH= 7.2); two earthworms were subsequently placed in 50 g of each soil sample, which was then kept for two days in the dark at approximately 10 °C. Afterwards earthworm coelomocytes were collected according to the extrusion method of Eyambe et al.[2] and subjected to an adapted SCGE-procedure (20 minutes alkali treatment; 30 minutes electrophoresis; 300 mA, 25 V). Fifty cells were investigated per dose. As illustrated in Figure 1, a clear dose-response relationship was obtained. For a concentration of 200 pg/g soil, expressed as sum of the concentrations of the seventeen congeners, the SCGE analysis was found impossible because of the high toxicity.

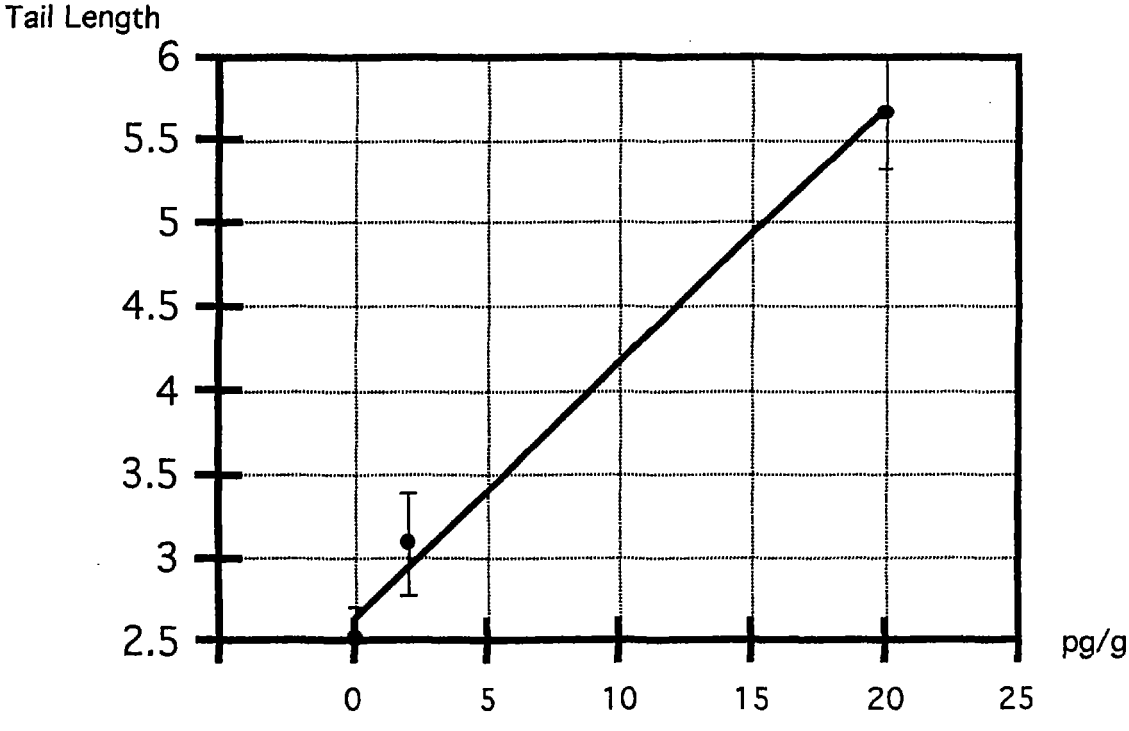

Figure 1 Earthworm SCGE-test after exposure to soils spiked with a mixture of the 'dirty seventeen' PCDD/PCDF congeners: comet tail length in function of PCDD/PCDF concentration in the soil (pg/g dry weight, expressed as sum of the concentrations of the seventeen congeners); the bars represent standard errors.

b. Exposure of earthworms to soils spiked with a dioxin-containing extract from a municipal waste incinerator (MWI) condensate

A large, homogeneous and representative sample of MWI emission gases was obtained by continuous sampling during a period of five days and nights, in which period the incinerator burned 552 tons of domestic waste with an emission of 5.960.000 Nm^3 gas on a dry basis. Sampling of 285 Nm^3 was performed, resulting in 35 l of condensate, according to a standard methodology. This condensate was extracted three times with CH_2Cl_2 resulting in 3.9 l of extract. Table 1 shows the concentrations of PCDDs and PCDFs in the extract; the isomer-specific analysis was performed using capillary gas chromatography - high resolution mass spectrometry (GC-HRMS) after purification of the extract by adsorption chromatography on an alumina column.

PCDD		PCDF	
congener	concentration (pg/ml extract)	congener	concentration (pg/ml extract)
2,3,7,8-T_4CDD	4.11	2,3,7,8-T_4CDF	28.4
1,2,3,7,8-P_5CDD	46.7	1,2,3,7,8-P_5CDF	69.8
1,2,3,4,7,8-H_6CDD	33.8	2,3,4,7,8-P_5CDF	124
1,2,3,6,7,8-H_6CDD	85.1	1,2,3,4,7,8-H_6CDF	152
1,2,3,7,8,9-H_6CDD	40.4	1,2,3,6,7,8-H_6CDF	166
1,2,3,4,6,7,8-H_7CDD	523	1,2,3,7,8,9-H_6CDF	17.1
OCDD	1080	2,3,4,6,7,8-H_6CDF	196
		1,2,3,4,6,7,8-H_7CDF	841
		1,2,3,4,7,8,9-H_7CDF	129
		OCDF	559

Table 1 Concentrations of PCDDs and PCDFs in the extract of a municipal waste incinerator condensate, which was used for earthworm SCGE-tests.

Prior to the SCGE test the extract was transferred to hexane in order to eliminate the toxic solvent. Then appropriate dilutions were made and applied to standard soil samples, which were subjected to a further treatment and used in an SCGE-test as described above. The results have been summarized in Figure 2 and again demonstrate a clear dose-response relationship.

BENEFITS AND PROBLEMS

The two examples described above indicate that the earthworm comet test may be useful for biomonitoring dioxin-related pollution, important benefits being the low cost and rapidity of the test (results may be obtained within a couple of days). Besides for monitoring, it may well be

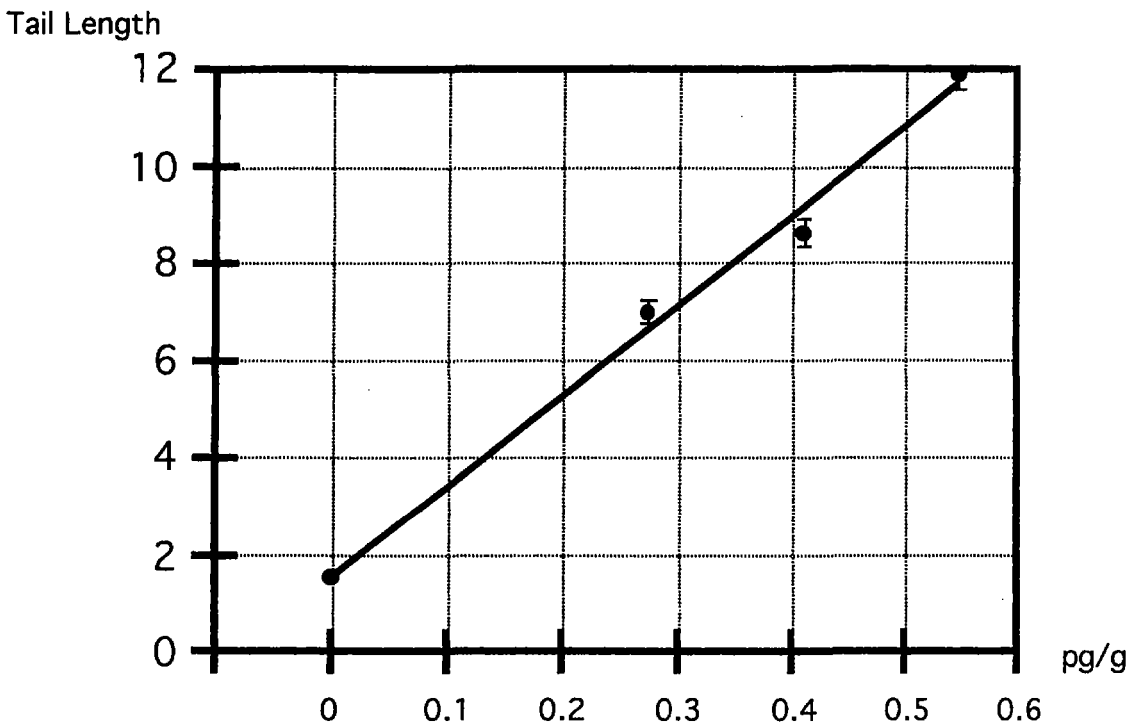

Figure 2 Earthworm SCGE-test after exposure to soils spiked with a dioxin-containing extract from a municipal waste incinerator condensate; for further explanation see Figure 1.

envisaged that the test could be a suitable tool for the evaluation of toxic equivalency factor schemes, at least as far as genotoxicity is concerned. Yet, some problems still need to be solved. For example, although dose-response relationships were found to be linear in both tests described above, the doses applied (expressed as sum of the concentrations of the 17 PCDD/PCDF per gram soil) for approximately the same comet tail lengths differed about two orders of magnitude. This undoubtedly is due to the presence of other (geno)toxic compounds in the extract from the municipal waste incinerator condensate. The lack of specificity could be deduced similarly from the results of another SCGE experiment, performed on earthworms which resided for two days in soils obtained in the vicinity of an illegal metal reclamation site. In this case microscope slides obtained at dioxin doses above 30 pg/g (expressed as sum of the concentrations of the 17 PCDD/PCDF) displayed a starry sky of fluorescent spots rendering any adequate lecture of the slides impossible. This is probably due to the abundance of heavy metals in these soils. Thus, the earthworm comet assay as presently applied is clearly not dioxin specific and may be too sensitive. Current research is therefore focusing on the adaptation of the test so as to make it meet better the requirements for dioxin biomonitoring and effect studies.

REFERENCES

1 Singh N.P., McCoy M.T., Tice R.R., Schneider E.L. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 1988: 184-191

2 Eyambe G.S., Goven A.J., Fitzpatrick L.C., Venables B.J., Cooper E.L. A non-invasive technique for sequential collection of earthworm (Lumbricus terrestris) leukocytes during subchronic immunotoxicity studies. Laboratory Animals 25, 1991: 61-67.