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In situ evaluation of the genotoxic potential of the river Nile: II. Detection of DNA strand-breakage and apoptosis in *Oreochromis niloticus niloticus* (Linnaeus, 1758) and *Clarias gariepinus* (Burchell, 1822)

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ABSTRACT

This work is part of a wider eco-toxicological study proposed to evaluate the biological impact of contaminants along the whole course of the river Nile, Egypt. Here we present data on the presence of DNA strand-breaks and apoptotic cells assessed by use of comet and diffusion assays in erythrocytes of Nile tilapia (*Oreochromis niloticus niloticus*) and African catfish (*Clarias gariepinus*). The results showed high degrees of DNA damage and increased frequencies of apoptotic nuclei in blood of fish collected from downstream compared with those sampled from upstream river Nile. Qualitative analysis revealed a shift in the frequency of DNA-damage classes towards higher damage levels correlating with the increasing pollution gradient. The degree of DNA damage measured by use of comet assay and diffusion assay exhibited seasonal variations. Both fish species showed significant increases in DNA damage during the summer. The results of our study indicated that the alkaline comet assay seems to be a useful technique for in situ genotoxic monitoring. At the same time the diffusion assay is sensitive enough to detect low frequencies of apoptotic nuclei. The results reveal species-specific differences in sensitivities, suggesting that Nile tilapia may serve as a more sensitive test species compared with the African catfish. Based on the outcome of the comet and diffusion assays, it can be concluded that the water quality of the river Nile with respect to the presence of genotoxic compounds needs to be improved, especially in its estuaries. As far as we know this is the first time that the comet and diffusion assays are used for genotoxic monitoring of the river Nile.

1. Introduction

The river Nile is the principal fresh-water resource for Egypt, meeting nearly all demands for drinking-water, irrigation, and industry [1,2]. For this reason, continuous monitoring for quality parameters is necessary. Despite the existence of relevant legislation, the pollution of the river Nile continues consequence of increasing agricultural, industrial and domestic effluents. While the quality of most of the Nile's water is within acceptable levels, there are several hot-spots of pollution, mostly found at certain sites along its course. Compounds present in polluted water are capable of causing biological alterations that can affect particular populations and entire ecosystems [3]. Some pollutants are highly persistent and have mutagenic and/or clastogenic properties. Because of the continued production and release of these pollutants into the aquatic environment, the investigation of the genotoxic potential of inland and coastal waters has become a major task in the monitoring of environmental pollution [4]. The interaction of genotoxic contaminants with DNA causes various genetic disturbances, which are often irreversible and can be transmitted to the next generations [5–7]. The loss of DNA integrity may determine the induction of mutations, chromosomal aberrations, birth defects and cancer in vertebrates [8,9]. Therefore, there is a great interest in assessing the impact of genotoxic compounds released into the aquatic environment.

The assessment of genotoxic potential in surface water is one of the main tasks of environmental monitoring to control pollution [3]. The analysis of environmental genotoxicity provides early warning signals of adverse long-term effects of the contamination [7]. DNA damage, such as strand breaks, has been proposed as a sensitive indicator of genotoxicity and an effective biomarker in environmental bio-monitoring studies [9,10]. The comet assay is

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currently the most widely employed method in eco-toxicology to detect DNA lesions. The alkaline comet assay is capable of detecting a wide variety of DNA damage, such as DNA single-strand breaks, double-strand breaks, oxidatively induced base damages, alkali-labile sites, and sites undergoing DNA repair [11–13]. The popularity of this test is a result of its sensitivity, relatively low costs, simplicity and time efficiency due to automatic scoring of the comets by use of image-analysis software [14–16]. It also has been employed to visualize DNA degradation due to apoptosis [17–19]. If the damage produced reaches a high level, it can finally lead to cell apoptosis [20]. As the frequency of apoptosis increases, the percentage of DNA in the tail comet increases until it disappears from the gel [13,21]. The DNA-diffusion assay, a modified version of the comet assay, allows the detection of apoptosis in single cells [19]. The diffusion assay described here is a simple, sensitive, and rapid method for estimating apoptosis in single cells.

In eco-toxicological studies it is essential to assess the toxic response of indigenous fauna as indicator of environmental pollution [3,22]. Fish are often used as bio-indicators because they play a several roles in the trophic web, bio-accumulate toxic substances, and respond to low concentrations of contaminants [22–24]. Estimation of DNA damage in fish can be carried out with a variety of tissue samples. Erythrocytes are the first choice because fish blood ensures great homogeneity of cells for comet studies [25,26]. Nile tilapia and African catfish are representative species of river Nile with high economic value. Both species can move along the entire water column, being a versatile indicator species. The ecological significance of the selected species could confirm their value as indicator species, and the wide zoogeographic distribution of Nile tilapia and African catfish could enable the comparison among different rivers by means of in situ monitoring.

In contrast to controlled laboratory studies, assessment of genotoxicity in aquatic organisms in their natural environment is a complicated task, mainly because of the relatively low levels of genotoxicants and the existence of multiple potentially genotoxic pollutants, often encountered as complex mixtures [27]. Although the comet assay has been successfully applied on several fish species exposed to genotoxic agents in vivo and in vitro [11,28–30], there are only a few aquatic bio-monitoring studies that use the comet assay in field-sampled fish [31,32]. To our knowledge, the genotoxicity of water from the river Nile has never been studied. Our previous work [2] made a first attempt to assess genotoxicity of the river Nile. Therefore, as a follow-up, we here present an investigation of DNA strand-breakage and apoptosis in fish, and assess the feasibility of using the comet and diffusion assays as indicators of genotoxins under actual field conditions. Assessment of DNA damage was performed in peripheral blood erythrocytes of African catfish and Nile tilapia collected from whole course of the river Nile. The information generated here will contribute to the utilization of the selected species for genotoxic bio-monitoring in polluted water.

2. Material and methods

2.1. Sampling sites

Six sites were selected along the whole course of the river Nile from its spring at Aswan to its estuaries at Rosetta and Damietta (Fig. 1).

2.2. Water quality assessment

Water-quality criteria [electrical conductivity, pH, water temperature, chemical oxygen demand (COD), total organic carbon (TOC), total solids (TS), ammonia (NH_3), nitrate (NO_3), orthophosphate (o-PO_4), chloride (Cl), fluoride (F), sulfate (SO_4), phenolics (Phenol)] of the chosen sites were monitored bimonthly during the period from July 2009 to June 2010. Total Pb, Cu, Cr, Hg, and Cd were measured using graphite furnace AA (GFAA) spectroscopy. Sampling and assessment of water quality were done according to the traditional manual methods [33]. Data on the selected sites are shown in Table 1.

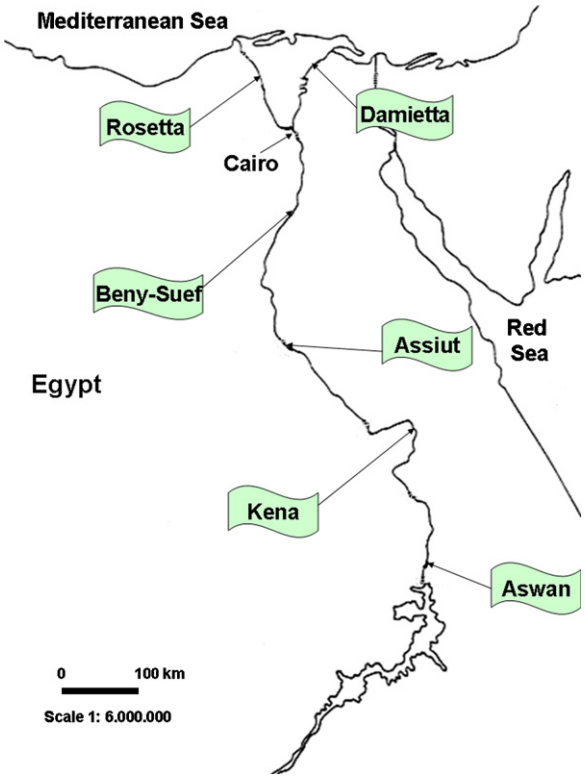

Fig. 1. Map showing the sampling sites along the whole course of the river Nile, from its spring at Aswan to its estuary at Damietta and Rosetta branches.

2.3. Fish sampling

Nile tilapia (*Oreochromis niloticus niloticus*) and African catfish (*Clarias gariepinus*) were caught bimonthly by gill net from the selected sites during the period from July 2009 to June 2010 (72 specimens from each species; body-weight ranging from 240 to 290 g for Nile tilapia and from 280 to 350 g for African catfish). Peripheral blood was collected by cardiac puncture with heparinized syringes from each fish as described by Osman et al. [2] for comet and diffusion assays. Blood samples were kept on ice and immediately processed for genotoxicity. Erythrocytes were processed for the evaluation of DNA integrity and apoptosis by comet assay and diffusion assay under yellow light.

2.4. Comet assay

The alkaline comet assay was performed according to the basic procedure of Singh et al. [34] considering the modification of Osman et al. [30] because of the unique characteristics of the blood. Heparinised blood was immediately diluted 50-fold in phosphate-buffered saline (PBS) [35]. Five μl of each diluted blood sample was added to 95 μl of 0.5% (w/v) low-melting agarose and the mixture was added onto a frosted microscope slide pre-coated with 1% (w/v) of normal-melting agarose and covered with a cover slip. The slide was incubated at 4 °C for 15 min to allow solidification and was subsequently coated with an additional layer of 0.5% low-melting agarose. After solidification at 4 °C for 20 min, the embedded cells were lysed in lysing buffer [2.5 M NaCl, 100 mM NaEDTA, 10 mM TRIS base, pH 10, 1% Triton X-100, 10% DMSO] at 4 °C for 120 min. After a 30 min incubation in electrophoresis buffer [300 mM NaOH, 1 mM EDTA, pH ≥ 13] electrophoresis was carried out at 20 V and 300 mA for 30 min. Subsequently, neutralization was performed in three washing steps in 0.4 M Tris–HCl (pH 7.5). To visualize DNA strand breaks, slides were stained with ethidium-bromide solution [20 μg/ml] for 10 min, and images were captured at 400× magnification by use of an Olympus fluorescence microscope (VANOX, AHBT3, model BH2-RFCA; Olympus America, Melville, NY, USA) and a color video camera (Olympus DP 20). DNA strand-breakage was quantified as the amount of fluorescence in the comet tail (% DNA in comet tail;% tail-DNA) with TriTek CometScore™ (Freeware image-analysis software v1.5). The percentage of DNA in the comet tail–assessed as optical density at 515–560 nm – was calculated for each nucleus [36]. Considering 100 nuclei, four replicates per site were analyzed (n = 2400 for each fish species). Calculations are means per replicate.

For qualitative evaluations, nuclei were categorized, according to the degree of damage (using % tail-DNA) based on the criteria of Anderson et al. [37] and Mitchelmore and Chipman [12], into five classes: undamaged nuclei (% tail-DNA ≤ 10%),