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“If it weren't for the last minute,
a lot of things wouldn't get done.”
Michael S. Taylor

I dedicate this work to my mother and father
for raising me the way they did.

Resumo

O Estuário do rio Sado, localizado na costa oeste de Portugal, é maioritariamente classificado como uma reserva natural. Contudo, as suas águas sofrem a influência de diversas fontes de poluição, tais como, poluição urbana e industrial, proveniente da cidade de Setúbal na margem Norte, e poluição associada ao tráfego marítimo, exploração mineira e actividades agrícolas intensivas. Este estuário, pela sua riqueza em espécies aquáticas, é também considerado um local privilegiado para actividades de pesca, efectuadas pelas populações locais, que não só consomem, mas também comercializam o produto da sua pesca. Por outro lado, as águas do estuário também podem ser usadas na agricultura, já que, na margem Sul, existem pequenas hortas e vastas áreas de arrozais. Estudos anteriores revelaram a existência de diversas classes de contaminantes nos sedimentos deste estuário, incluindo metais, pesticidas e hidrocarbonetos aromáticos policíclicos, cujos efeitos adversos em organismos vivos são bem conhecidos. Estes compostos podem ser acumulados nas partes edíveis de espécies aquáticas estuarinas, ou em produtos agrícolas, e entrar na cadeia alimentar humana, tornando-se um problema de saúde pública. Este estudo insere-se num projecto mais abrangente que tem como objectivo último a avaliação do risco ambiental, incluindo ecológico e para a saúde humana, associado ao ambiente bentónico estuarino e complementado com a análise de uma população localizada na margem Sul do Estuário do Sado. O presente estudo teve como objectivo específico a avaliação do potencial citotóxico e genotóxico de sedimentos provenientes do Estuário do Sado, através do ensaio do vermelho neutro, e dos ensaios do cometa e do micronúcleo, respectivamente, numa linha celular humana (HepG2).

Foram realizadas colheitas de amostras de sedimentos em quatro locais distintos e potencialmente contaminados do Estuário do Sado, dois próximos da margem Norte (P e C) e outros dois junto à margem Sul (E e A). Incluiu-se também, como controlo positivo, uma amostra da margem Norte deste estuário (F), previamente caracterizada pelo elevado nível de contaminação e propriedades genotóxicas em espécies aquáticas. Como área estuarina de referência (não contaminada) seleccionou-se o Estuário do rio Mira (Oeste Portugal), onde foram colhidas: uma amostra de referência (Mf), e uma amostra mais a jusante, junto a uma aquacultura (M). As várias amostras de sedimentos foram sujeitas a uma extracção mecânica com uma mistura de metanol:diclorometano (1:2), permitindo a extracção da grande maioria dos contaminantes orgânicos e

inorgânicos, que foram posteriormente recuperados em DMSO, para os ensaios celulares.

Avaliou-se a citotoxicidade dos vários extractos em células HepG2 expostas durante 2, 4 e 48h a um intervalo de concentrações entre 0,1 e 20µl/ml de meio de cultura, recorrendo-se ao ensaio do vermelho neutro. A caracterização do efeito genotóxico dos vários extractos foi realizada utilizando a versão alcalina do ensaio do cometa na mesma linha celular, e, com vista à quantificação adicional dos danos oxidativos existentes no DNA, em particular purinas oxidadas, recorreu-se também ao mesmo ensaio com tratamento com a enzima FPG. O ensaio do micronúcleo em células com bloqueio da citocinese foi utilizado apenas preliminarmente para estudar os efeitos lesivos ao nível cromossómico dos extracto F, C e M.

O período de exposição mais curto, utilizado numa primeira fase para testar as amostras F, C e M, não revelou a existência de um efeito citotóxico marcado, tendo-se optado por utilizar uma exposição mais prolongada de 48h, para todas as amostras em estudo. No que diz respeito ao efeito citotóxico dos extractos de sedimentos do Estuário do Sado, observou-se uma relação dose-resposta para as amostras F, P, e E, e calculou-se o IC_{50} para cada extracto a partir da equação da curva. Em contraste, não foi observada citotoxicidade valorizável para as amostras A e C. Verificou-se que o potencial citotóxico do extracto P, correspondente à amostra colhida junto à margem Norte, é comparável ao da amostra usada como controlo positivo (F), enquanto que os extractos das amostras colhidas junto à margem Sul mostram um menor efeito (E) ou mesmo ausência de efeito (A) na viabilidade celular. Relativamente aos extractos obtidos das amostras do Estuário do Mira, a amostra M produziu efeitos citotóxicos, ao contrário da amostra Mf, que não causou morte celular. Os resultados de citotoxicidade determinaram também o intervalo de concentrações a utilizar para cada extracto nos ensaios de genotoxicidade, de forma a evitar o aparecimento de resultados falsamente positivos.

A análise dos efeitos lesivos no DNA, após a exposição de células HepG2 a várias concentrações dos extractos, mostrou que a concentração mais elevada das amostras P e E do estuário do Sado e da amostra M do Mira, causaram uma indução significativa de lesões no DNA, comparativamente ao controlo de solvente. O mesmo se observou para a amostra F, o controlo positivo do Sado. Não obstante, o tratamento com FPG fez aumentar o nível de lesões das amostras P, E, A, e M, e do controlo positivo F, indicando a existência de danos oxidativos adicionais no DNA. Não foi observada

genotoxicidade significativa para a amostra C, em qualquer condição testada. Tendo em consideração o nível de lesões de DNA induzido pela concentração mais elevada de cada extracto, as amostras podem ser ordenados por ordem crescente da sua potência genotóxica da seguinte forma: P > F > E > M > Mf > A > C. O mesmo tipo de análise, mas aplicado ao nível de lesões oxidativas induzidas no DNA mostra um ordenamento diferente das amostras: E > A > M > P > F > Mf > C, colocando em evidência a capacidade das amostras E e A de induzirem danos oxidativos.

Recorrendo ao ensaio do micronúcleo, apenas para os extractos F, M e C, não foi possível observar um aumento significativo da frequência de micronúcleos, com o aumento da concentração.

As diferenças observadas, quer no efeito citotóxico quer genotóxico, entre os extractos preparados a partir das diferentes amostras de sedimentos, poderão ser atribuídas, principalmente, a três tipos de factores: i) factores relacionados com os diferentes tipos de contaminação, antropogénica ou natural, que influenciam cada local de amostragem; ii) diferenças nas pressões hidrodinâmicas de cada local, relacionadas com correntes e marés; iii) diversidade de características granulométricas dos sedimentos que afectam a capacidade de adsorção e retenção de contaminantes. No Estuário do Sado, observou-se uma diferença entre as amostras colhidas numa área de influência industrial e urbana (F e P), e numa área com influência agrícola (A e E). As diferenças detectadas sugerem um tipo ou nível de contaminação diferente, dado que, as amostras F e P têm uma relação mais directa entre a citotoxicidade e a genotoxicidade, e as amostras A e E, embora não sejam particularmente citotóxicas, foram capazes de induzir níveis mais elevados de danos oxidativos no DNA. Este facto, poder-se-á dever à presença de HAPs, PCBs ou metais nas amostras F e P, e à presença de pesticidas e metais nas amostras A e E, sugerido quer pelo tipo de pressões antropogénicas associadas, quer por dados de alguns estudos anteriores em sedimentos deste estuário. Os resultados de cito e genotoxicidade sugerem que a amostra C terá níveis de contaminação muito baixo ou mesmo inexistentes, o que possibilita a utilização desta amostra como referência do Estuário do Sado, para ensaios futuros.

Quanto ao Estuário do Mira, observaram-se dois tipos de resposta, quer na citotoxicidade, quer na genotoxicidade, o que parece reflectir dois tipos, ou níveis, de contaminação diferentes. Os resultados obtidos para a amostra M poderão estar associados à existência de resíduos provenientes de uma aquacultura, perto do ponto de amostragem, assim como à presença de metais de origem natural. A amostra Mf não

revelou efeitos biológicos valorizáveis, pelo que parece corresponder a uma área não contaminada e, consequentemente, ser uma boa amostra de referência.

Em suma, os resultados obtidos reflectem o tipo e nível de contaminação diferencial presente no Estuário do Sado, que advêm de fontes de contaminação e de pressões ambientais diferentes, comparativamente ao obtido no Estuário do Mira. Dada a possibilidade destes contaminantes entrarem na cadeia alimentar humana, através da bioacumulação em espécies aquáticas ou produtos agrícolas, este estudo é imperativo e deverá ser prosseguido, no sentido de estabelecer uma associação entre a contaminação dos sedimentos estuarinos e os possíveis efeitos na saúde humana, demonstrando ser uma temática de saúde pública com importância na biomonitorização ambiental.

Palavras chave: Sedimentos; contaminantes; genotoxicidade; citotoxicidade; estuário.

Abstract

The Sado Estuary (W Portugal) is affected by various sources of pollution, associated with the existence of an urban center, heavy-industry, mining activities and agriculture. It also remains a privileged site for fishing activities that are responsible for the supplying of consumable resources either locally or externally. Previous studies revealed sizable amounts of contaminants in the estuary sediments, namely metals, pesticides and polycyclic aromatic hydrocarbons. These compounds can be absorbed and accumulated in the edible parts of estuarine species and in local agricultural products, thus entering the human food chain and posing a public health problem. This study aims to assess the cytotoxic and genotoxic effects of sediments from the Sado Estuary in a human cell line, in order to contribute to hazard identification. Sediments were collected in 4 distinct fishing sites (P, E, A, C) of the Sado Estuary; a reference (Mf) and a potentially contaminated sample (M) from a different estuary (Mira Estuary, W Portugal) were also included. Total organic and inorganic contaminants were extracted with a mixture of methanol:dicholomethane and recovered in DMSO. HepG2 cells were exposed for 48h to several concentrations of each extract. Cytotoxicity was measured by the neutral red assay and genotoxicity by the comet assay. A dose-related decrease in cell viability was observed for extracts P, E and M, indicating sediment contaminant-driven cytotoxicity, whereas no significant cytotoxicity was observed for extracts A, C and Mf. A significant increase in the level of DNA damage was observed following cells exposure to extracts P, E, A and M. Also, increased genotoxicity was observed following treatment with the endonuclease FPG, suggesting the induction of oxidative DNA damage. No significant genotoxicity was induced by extracts C and Mf. The differential cytotoxicity and genotoxicity observed in samples from the northern and southern areas of the Sado Estuary probably reflects the pollution from heavy-industrial and urban area vs. intense agricultural area, respectively. Thus, the data suggest the existence of an inherent environmental, ecological and human health, risk associated with the Sado Estuary sediment contamination that must be further assessed.

Keywords: Sediments; contaminants; genotoxicity; cytotoxicity; estuary.

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Abbreviations List

ALS – Alkali-Labile Sites

ATCC – American Type Culture Collection

BaP – Benzo[*a*]pyrene

BNC – Binucleated Cell

CBMN – Cytokinesis-Block Micronucleus

CBPI – Cytokinesis-Block Proliferation Index

CHO-K1 – Chinese Hamster Ovary Cell Line

CYP1A1 – Cytochrome P-4501 A1

DDT – Dichlorodiphenyltrichloroethane

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

DSB – Double Strand Break

EDTA – Ethylenediamine Tetraacetic Acid

EMS – Ethyl Methanesulfonate

ENA – Erythrocytic Nuclear Abnormalities

FBS – Fetal Bovine Serum

FF – Fine Fraction

FPG – Formamidopyrimidine DNA Glycosylase

GC-MS – Gas Chromatography – Mass Spectrometry

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid

HepG2 – Human Hepatocellular Liver Carcinoma Cell Line

HPLC – High-Performance Liquid Chromatography

IARC – International Agency for Research on Cancer

IC_{50} – Inhibitory Concentration at 50%

ICP-MS – Inductively Coupled Plasma Mass Spectrometry

LDH – Lactate Dehydrogenase

LMP – Low Melting Point

MN - Micronucleus

MNi – Micronuclei

MNC – Multinucleated Cell

MTT – Methyl Tetrazolium

NBUD – Nuclear bud

NMP – Normal Melting Point

NPB – Nucleoplasmic Bridge

NR – Neutral Red

PAH – Polycyclic Aromatic Hydrocarbon

PBS – Phosphate Buffered Saline

PCB – Polychlorinated Biphenyl

PEL – Probable Effect Level

PLHC-1 – *Poeciliopsis lucida* Hepatocellular Carcinoma Cell Line

RI – Replication Index

ROS – Reactive Oxygen Species

RTL-W1 – Rainbow Trout Liver Cell Line

SD – Standard Deviation

SE – Standard Error

SQG-Q – Sediment Quality Guideline Quotients

SSB – Single Strand Break

TEL – Threshold Effect Level

TOM – Total Organic Matter

UV – Ultra-Violet

INTRODUCTION

1. Estuarine Sediment Contamination

1.1. Overview

Rivers, lakes and seas receive large quantities of waste water from industrial, agricultural and domestic sources. In fact, many industrial wastes and effluents, have been shown to be responsible for contaminant discharges [1]. Estuaries are a good example of this problem because they are commonly subjected to anthropogenic pressures from different sources such as industry, urbanism, maritime traffic, tourism, agriculture and fishing activities, located along their margins [2]. The persistent contaminants in surface water can be genotoxic, mutagenic and carcinogenic compounds that in a complex environmental mixture may have adverse effects on the indigenous biota exposed, and, more importantly, on the human health [3]. Particularly, water pollution can become a serious public health problem because these contaminated surface waters may be used as a source of drinking water and for agriculture or recreational activities. In addition to direct exposure, the local edible aquatic species may bioaccumulate these contaminants and enter the human food chain, becoming another source of human exposure and of concern regarding public health [4].

An estuary is a semi-enclosed maritime area with limited self renewal capability which makes it particularly capable of retaining contaminants from different sources. These contaminants can also be altered in terms of bioavailability and toxicity due to the variation of abiotic factors such as salinity, pH or temperature. In light of this, pollution in these environments has become an important issue [4]. Also, it is now considered that estuary sediment contamination is a major pressure on ecosystem health [2]. In fact, aquatic sediments can become a sink for hydrophobic environmental mutagens that are adsorbed by suspended particulate matter and allowed to accumulate, settle and incorporate into the bottom sediments. There has been a growing concern towards these aquatic sediments for they may become reservoirs of mutagenic hazard, and the adsorbed mutagens can be continually resuspended into the water column, making them available to the local biota [1].

Sediments are complex matrices composed of organic matter, particulate mineral material and inorganic material of biogenic origin. Each of these components varies in size and composition, and influences the sediments' ability to adsorb aquatic contaminants, their bioavailability and toxicity. It is suggested that most contaminants

are predominantly associated with fine deposits that are rich in organic matter. Also, metals from natural or anthropogenic sources are deposited and associated with fine-grained sediments. Nevertheless, the association between contaminants and aquatic sediments will depend on the physiochemical properties of the contaminants and sediments, abiotic factors, as well as other environmental conditions, such as hydrodynamic pressures that may be responsible for the contaminants distribution and resuspension [1, 5].

The genotoxic assessment of aquatic sediments can be performed in whole sediments, interstitial water, sediment elutriates or sediment extracts. Rather than only applying sediment chemical analysis, focus is being drawn to the use of bioassays in order to investigate the genotoxic hazards of aquatic sediments. Examples of different approaches for the study of sediment contamination and toxicity, including genotoxicity, are presented in Table 1. In fact, the genotoxic effects of aquatic sediments have already been demonstrated on the biota inhabiting a contaminated site [1]. Nevertheless, even coupled with chemical analysis, bioassays can be limited in attributing the mutagenic hazard of aquatic sediments to a given contaminant, since there is a poor understanding of the behavior of mutagens in such a complex environmental mixture. In fact, additive, synergistic and antagonistic effects have been proven to occur in these contaminant mixtures [6, 7]. Therefore, careful considerations should be made when assessing the potential risk of aquatic sediments so as not to underestimate it [1].

Table 1 – Examples of different methodological approaches and outcomes from several studies on marine sediment contamination and toxicity.

Country	Methodological Approach	Outcome	Reference
Australia	Measurement of metals in fish skeletal tissue and seston samples	High levels of Cu, Cd and Pb associated with heavy industry and micronucleus induction	Edwards *et al.* (2001) [8]
	Micronucleus assay in marine fish (*Aldrichetta forsteri* and *Sillago schomburgkii*)		Hamann *et al.* (1999) [9]
Brazil	Comet assay in RTL-W1 cells Micronucleus assay in Nile tilapia (*Oreochromis niloticus*) blood cells.	↑ DNA damage ↑ micronucleus formation	Rocha *et al.* (2009) [10]
China	MTT assay Comet assay in flounder cell line Zebrafish embryo test PAH analysis by GC-MS	Dose-related cytotoxicity Dose-related genotoxicity Time-dependent genotoxicity Dose-related teratogenicity Correlation between PAH content and sediment toxicity	Yang *et al.* (2010) [11]
Croatia	Comet assay in PLHC-1 cells	↑ DNA strand breaks	Šrut *et al.* (2010) [12]
France	PAH analysis by HPLC Salmonella mutagenicity test on sediments Micronucleus assay in CHO-K1 cells Comet assay in CHO-K1 cells	16 quantified PAHs positive mutagenic activity ↑ micronucleus frequency ↑ DNA damage	Aouadene *et al.* (2008) [13]
Germany	Comet assay in RTL-W1 cells and zebrafish (*Danio rerio*) embryo cells	↑ DNA damage	Seitz *et al.* (2008) [14]
Germany	Chemical analysis by GC-MS Comet assay in carp (*Cyprinus carpio* L.) cell line Zebrafish embryo test	Quantified PAH and PCB positive teratogenic effects ↑ DNA damage	Kammann *et al.* (2004) [15]
Korea	Comet assay in flounder (*Paralichthys olivaceus*) blood cells PAH analysis by GC-MS	↑ DNA strand breaks 24 quantified PAHs	Woo *et al.* (2006) [16]
Portugal	Comet assay in fish blood (*Solea Senegalensis*) Erythrocytic nuclear abnormalities (ENA) scoring Chemical analysis by ICP-MS, GC-MS	↑ DNA damage ↑ ENA Quantification of PAHs, PCBs, metals, DDTs.	Costa *et al.* (2011) [17]
United Kingdom	Mutatox^{TM} bioassay with S9 metabolization Extract fractionation by HPLC Chemical analysis by GC-MS	positive mutagenic activity by quantified PAHs and other contaminants	Thomas *et al.* (2002) [18]

↑ - effect increase. GC-MS – Gas Chromatography – Mass Spectrometry. HPLC – High-Performance Liquid Chromatography. ICP-MS – Inductively Coupled Plasma Mass Spectrometry.

1.2. Main groups of sediment contaminants

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants that originate during the incomplete combustion of organic material. In particular, industrial processes that involve the pyrolysis or combustion of coal are major sources of PAHs. They are organic compounds that contain only carbon and hydrogen, and are comprised of two or more fused aromatic ring. Found in air, water, soils and sediments, generally at trace levels except near their sources, the environmental and occupational exposure to these contaminants has been linked to mutagenesis and cancer. In fact the International Association for Research on Cancer (IARC) as classified many of these compound as probable carcinogens or possible

carcinogens to humans, groups 2A and 2B, respectively (Table 2) [19]. The majority of these compounds require metabolic activation to electrophilic metabolites, through different metabolic pathways, in order to exert their mutagenic or carcinogenic effects [20]. Of particular interest among PAHs is benzo[*a*]pyrene (BaP), a ubiquitously environmentally distributed compound formed during the combustion of organic matter. Its carcinogenic and mutagenic effects have been extensively investigated and it is proposed that, during the metabolic process, via cytochrome P-4501 A1 (CYP1A1), BaP produces particular metabolites capable of causing oxidative DNA damage and form adducts with DNA [21, 22]. It is the only PAH, to date, to be classified by the IARC in group 1, *i.e.* as carcinogen to humans [23].

Table 2 – IARC carcinogenicity evaluation of several polycyclic aromatic hydrocarbons (Adapted from [19, 23]).

Common name	Group	Common name	Group
Acenaphthene	3	Dibenzo[*a*,*e*]fluoranthene	3
Acepyrene	3	13*H*-Dibenzo[*a*,*g*]fluorene	3
Anthanthrene	3	Dibenzo[*h*,*rst*]pentaphene	3
Anthracene	3	Dibenzo[*a*,*e*]pyrene	3
11*H*-Benz[*b*,*c*]aceanthrylene	3	Dibenzo[*a*,*h*]pyrene	2B
Benz[*j*]aceanthrylene	2B	Dibenzo[*a*,*i*]pyrene	2B
Benz[*l*]aceanthrylene	3	Dibenzo[*a*,*l*]pyrene	2A
Benz[*a*]anthracene	2B	Dibenzo[e,*l*]pyrene	3
Benzo[*b*]chrysene	3	1,2-Dihydroaceanthrylene	3
Benzo[*g*]chrysene	3	1,4-Dimethylphenanthrene	3
Benzo[*a*]fluoranthene	3	Fluoranthene	3
Benzo[*b*]fluoranthene	2B	Fluorene	3
Benzo[*ghi*]fluoranthene	3	Indeno[1,2,3-*cd*]pyrene	2B
Benzo[*j*]fluoranthene	2B	1-Methylchrysene	3
Benzo[*k*]fluoranthene	2B	2-Methylchrysene	3
Benzo[*a*]fluorene	3	3-Methylchrysene	3
Benzo[*b*]fluorene	3	4-Methylchrysene	3
Benzo[*c*]fluorene	3	5-Methylchrysene	2B
Benzo[*ghi*]perylene	3	6-Methylchrysene	3
Benzo[*c*]phenanthrene	2B	2-Methylfluoranthene	3
Benzo[*a*]pyrene	1	3-Methylfluoranthene	3
Benzo[*e*]pyrene	3	1-Methylphenanthrene	3
Chrysene	2B	Naphtho[1,2-*b*]fluoranthene	3
Coronene	3	Naphtho[2,1-*a*]fluoranthene	3
4*H*-Cyclopenta[*def*]chrysene	3	Naphtho[2,3-*e*]pyrene	3
Cyclopenta[*cd*]pyrene	2A	Perylene	3
5,6-Cyclopenteno-1,2-benzanthracene	3	Phenanthrene	3
Dibenz[*a*,*c*]anthracene	3	Picene	3
Dibenz[*a*,*h*]anthracene	2A	Pyrene	3
Dibenz[*a*,*j*]anthracene	3	Triphenylene	3

When compared with organic materials, metals are small molecules that are naturally present in the environment and are essential for the sustainment of life. Nevertheless, some of them may become toxic depending on their concentrations, oxidation state, complex form and mode of exposure. In fact, metals and their derivatives are not degraded by living organisms and may be accumulated up to hazardous levels, where they can interact with cellular macromolecules, metabolic and

transduction pathways and genetic processes (Figure 1). The IARC has classified some metals and metallic substances as carcinogens, and probable or possible carcinogens to humans (Table 3). They may be carcinogenic in the form of free ions, metal complexes or particles of metals [24]. It is suggested that oxidative stress may explain the mutagenic and carcinogenic effects of metals (Figure 1). Thus, through the formation of reactive oxygen species (ROS), metals can induce DNA damage indirectly [24, 25]. Previous studies have also revealed that carcinogenic metal compounds can often be comutagenic, promoting the mutagenicity of other genotoxic agents [24].

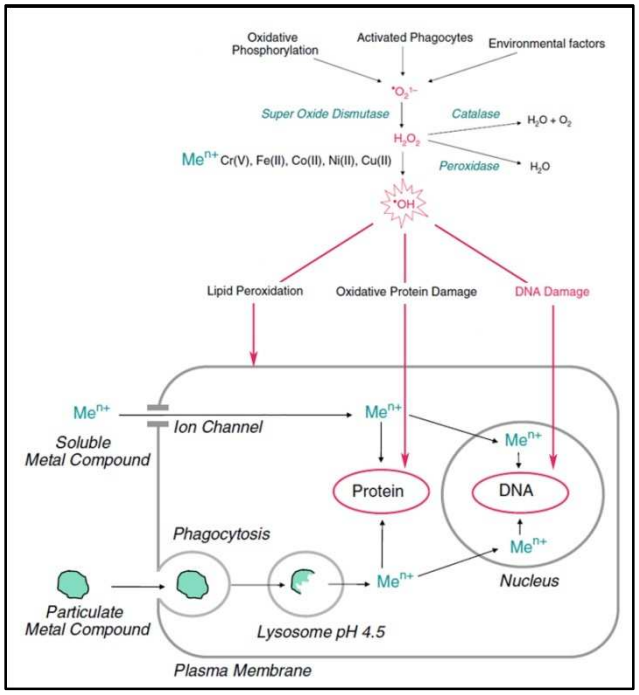

Figure 1 – Cellular uptake, intracellular distribution and binding of soluble and particulate metal compounds; induction of oxidative stress to macromolecules (Adapted from [24]).

Table 3 – Classification of metals and/or their compounds by the IARC (Adapted from [24]).

Subtances	Group
Antimony and its compounds	-
Antimony trioxide (Sb_2O_3)	2B
Antimony trisulfide (Sb_2S_3)	3
Arsenic and its compounds	1
Beryllium and its compounds	1
Cadmium and its compounds	1
Chromium metal	3
Chromium (VI) compounds	1
Chromium (III) compounds	3
Cobalt and its compounds	2B
Cobalt with tungsten carbide (hard metal)	2A
Gallium arsenide	1
Indium Phosphide	2A
Lead metal	-
Lead compounds	2A
Mercury and its compounds	2B
Nickel metal	2B
Nickel compounds	1
Rhodium	-
Selenium and its compounds	3
Vanadium and its compounds	-
Vanadium pentoxide (V_2O_5)	2B

Polychlorinated biphenyls (PCBs) are manufactured organic compounds used as dielectrics in transformers and capacitors, as cooling fluids, in hydraulic systems, pesticides and flame retardants, and as plasticizers in paints, copying paper, adhesives, sealants and plastics. The IARC classifies them in the 2A category as probable carcinogens to humans [26]. Even though their production has declined or has been banned in most countries, these compounds still persist in our environment. Previous studies have linked the exposure to PCBs to the formation of DNA adducts and DNA strand breaks but also to oxidative stress [27-29]. In fact, it is suggested that the metabolism of PCBs may produce metabolites capable of forming ROS and induce DNA strand breakage [30].

Dichlorodiphenyltrichloroethane (DDT) was a commonly used pesticide for insect control in many countries. Technical-grade DDT is a complex mixture of *para, para'*DDT, its isomers and related compounds. It is a highly persistent ubiquitous environmental contaminant found in food, soils and sediments, although its use has been forbidden many years ago. The IARC classifies this contaminant in group 2B as a possible carcinogen to humans [31]. It has been proposed that DDTs and their metabolites can induce cellular apoptosis, but that they may also induce oxidative DNA damage [32].

As previously mentioned, despite the fact that many of the compounds mentioned above have been shown to induce toxicity individually (particularly many PAHs and metals), several studies have shown that they can have additive, synergistic or antagonistic effects, when combined with the same type of contaminant or even with different types of contaminants present in complex mixtures [6, 7, 24, 25, 33]. From the mode of action of those chemicals, it is plausible that the formation of ROS, or oxidized metabolites, might be a common mechanism of induction of DNA damage, by metals [24, 25], PAHs [20, 34, 35], PCBs [29, 36], or DDTs [32]. There is a biologically tolerable level of free radicals that, when exceeded, can cause an elevated level of oxidative damage to biomolecules (such as lipids, proteins and nucleic acids) resulting in deleterious biological effects [29, 37].

2. Study Areas

2.1. Environmental Characterization

The Sado Estuary, located on the western coast of Portugal, is the second largest in the country, with an area of approximately 24 000ha (Figure 2). Even though most of the estuary is classified as a natural reserve it also plays an important role in the local and national economy. The northern margin of the estuary is characterized by an urban and heavy-industrialized area, located around the City of Setúbal. Along with the harbor-associated activities and the copper mines near the Sado, the major polluters are the industries involving pulp and paper, pesticides, fertilizers, processed food and shipyards [2, 38]. The southern margin is mostly characterized by the presence of small villages and by intense agricultural activities, particularly rice fields, as well as by the existence of the Troía Peninsula, an area of great touristic value. Fishing activities are also significant in the estuary, and the flatfish (*Solea senegalensis),* the cuttlefish (*Sepia officianalis*), the grouved carpet clam (*Ruditapes decussatus*), the crab (*Carcinus*

maenas) and the cockle (*Cerastoderma edule*) are aquatic species that are consumed by the local communities and may be distributed through economical channels along the country. Due to the presence of heavy-industry, urbanism, tourism, mining, agricultural activities and maritime traffic, the Sado Estuary is particularly subject to contamination from these different sources [2, 39]. For the above mentioned reasons, the Sado Estuary is a good example of a site where anthropogenic pressures and natural values compete with each other.

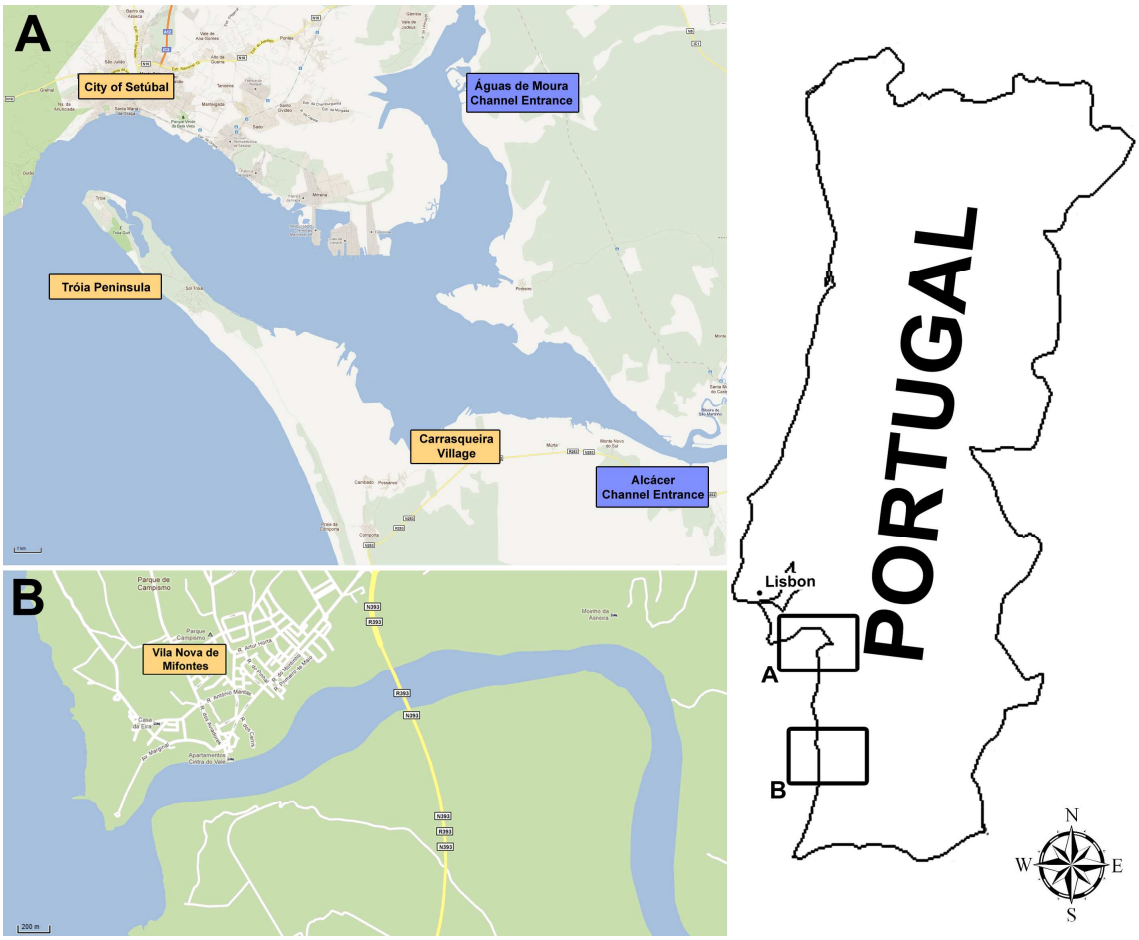

Figure 2 – Geographical location of the study area. **A** – Map of the Sado Estuary. **B** – Map of the Mira Estuary (Adapted from [40]).

In terms of hydrodynamics, the Águas de Moura northern channel of the estuary is characterized by weaker residual currents and shear stress, which allows locally introduced contaminants to settle and accumulate in sediments near the northern margin, rather than being transported away to the sea. The Alcácer southern channel is highly dynamic with tides being the main cause of water circulation. Nevertheless, the entrances to the Águas de Moura and the Alcácer Channels are shallow with tidal flats [2]. It is also suggested that the estuary's contamination is influenced by river flows carrying the present pollutants, particularly pesticides and fertilizers from the extensive

agriculture grounds upstream, which can settle near the southern margin of the estuary [17].

The Mira Estuary, also located in the western coast of Portugal (Figure 2), is being studied for its sediment contamination. Much like the Sado Estuary, it is comprised by a local population invested in agriculture and fishing activities, and by an urban area, the Vila Nova de Milfontes village. Little is known about this estuary's contamination state, but fewer contamination sources seem to be present in this area. The main potential sources of contamination are the agricultural activities, that are performed to a lesser extent than along the Sado Estuary, the urban discharges, and the aquaculture vivariums located along the estuary.

2.2. Characterization of contaminants and their biological effects in the Sado Estuary

Previous studies of the Sado Estuary have established a spatial distribution of several contaminants naturally present or locally produced and discharged. Sizable amounts of contaminants have been observed in the estuary sediments, namely PAHs, PCBs, metals and pesticides [2, 38, 39, 41]. Maximum and minimum metallic and organic contaminant concentrations, extrapolated from a combined historical set of values found in the Sado Estuary, can be seen in Tables 4 and 5, respectively.

Table 4 – Minimum and maximum metallic contaminant concentrations in sediments collected from the Sado and Mira Estuaries (Adapted from [42]).

	Metallic contaminants (ng.g^{-1} sediment dry weight)					
	Sado		**Mira**			
Compounds	**Min**	**Max**	**Min**	**Max**	**TEL**	**PEL**
Cd	0.4	**6.4**[a]	0.062	0.12	0.68	4.21
Pb	3.5	69	15	23	30.2	112
Zn	6.4	**507**[a]	34	57	124	271
Cu	1.5	**191**[a]	16	**30**[b]	18.7	108
Cr	1.3	44	34	44	52.3	160
As	2.4	37	14	**18**[b]	7.24	41.6
SQG-Q Metals	0.04	**1.08**[c]	0.16	0.24		

TEL – Threshold effect level; PEL – Probable effect level; SQG-Q – Sediment quality guideline quotient. The TEL and PEL were obtained from Macdonald *et al.* (1996) [43]. Calculation of SQG-Q according to Long and Macdonald (1998) [44]. [a]Concentration > PEL. [b]Concentration > TEL. [c]SQG-Q Maximim.

Ongoing studies on the Mira Estuary contamination have revealed different levels of contamination. Maximum and minimum metallic and organic contaminant

concentration values, found in the Mira Estuary, can also be seen on Tables 4 and 5, respectively.

Table 5 – Minimum and maximum organic contaminant concentrations in sediments collected from the Sado and Mira Estuaries (Adapted from [42]).

	Organic contaminants (ng.g^{-1} sediment dry weight)					
	Sado		**Mira**			
Compounds	**Min**	**Max**	**Min**	**Max**	**TEL**	**PEL**
acenaphthylene	0.79	2.38	~ 0	1.65	5.87	128
acenaphthene	0.73	**12.25**[a]	~ 0	1.41	6.71	88.9
fluorene	1.19	15.33	~ 0	4.32	21.2	144
phenanthrene	10.28	63.87	~ 0	45.52	86.7	544
anthracene	2.3	21	~ 0	8.79	46.9	245
fluoranthene	23.34	**345.24**[a]	~ 0	**161.7**[a]	113	1494
pyrene	21.51	**286.33**[a]	~ 0	118.2	153	1398
benzo[*a*]anthracene	3.7	**93.99**[a]	~ 0	58.63	74.8	693
chrysene	2.35	46.68	~ 0	24.73	108	846
benzo[*a*]pyrene	5.42	**126.76**[a]	~ 0	63.72	88.8	763
dibenzo[*a.h*]anthracene	0.66	**13.93**[a]	~ 0	**11.61**[a]	6.22	135
tPCB	0.8	11.97	0.0431	7.656	21.6	189
pp'DDD	0.37	0.71	~ 0	**1.9**[a]	1.22	7.81
pp'DDE	0.59	0.59	~ 0	1.18	2.07	374
pp'DDT	~ 0	**1.22**[a]	~ 0	0.041	1.19	4.77
SQG-Q Org	0.027	**0.118**[b]	1.52×10^{-5}	0.063		

TEL – Threshold effect level; PEL – Probable effect level; SQG-Q – Sediment quality guideline quotient. The TEL and PEL were obtained from Macdonald *et al.* (1996) [43]. Calculation of SQG-Q according to Long and Macdonald (1998) [44]. [a]Concentration > PEL. [b]SQG-Q Maximum.

As shown, this estuary displays, in general, a low level of contamination, and can therefore be considered as a reference site. Sediments from both estuaries have been recently studied for their contamination risk, through the calculation of sediment quality guideline quotients (SQG-Q) developed by Long and Macdonald (1998) [44], which revealed a moderate risk in the Sado Estuary, and a low risk in the Mira Estuary (Table 6) [42].

Table 6 – Global overview of sediment contamination risk. SQG-Q index values per class of contaminants (Adapted from [42]).

	Sado		**Mira**	
SQG-Q	**Min**	**Max**	**Min**	**Max**
SQG-Q Metals	0.04	1.08	0.16	0.24
SQG-Q DDT's	0.078	0.103	5.69×10^{-5}	0.074
SQG-Q PCB's	0.0042	0.063	2.28×10^{-4}	0.041
SQG-Q PAH's	0.0089	0.119	1.89×10^{-5}	0.058
SQG-Q org	0.027	0.118	1.52×10^{-5}	0.063
SQG-Q Total	0.025	**0.338**[a]	0.037	**0.092**[b]

SQG-Q – Sediment quality guideline quotient. [a]Maximum SQG-Q observed indicates moderate risk (SQG-Q > 0.1). [b]Maximum SQG-Q indicates low risk (highest SQG-Q < 0.1). Calculation of SQG-Q according to Long and Macdonald (1998) [44].

Assessment of metal contamination in the Sado Estuary revealed areas with high contamination and moderate ecological risk. The spatial distribution of different metals was revealed to be similar, and several contaminated sites were identified as “hotspots” close to their anthropogenic sources. These sites were identified as being in the northern channel, and can be associated with the different industries, shipyards and urban discharges of the City of Setúbal [2, 39]. The same sites have also been shown to be associated high organic loads [45]. The sediment contaminants were characterized in samples from this area, revealing levels, above the threshold, of As, Cr, Cu, Pb and Zn for metal and metalloid contaminants and of acenaphthene, fluoranthene, pyrene, benzo[*a*]anthracene and benzo[*a*]pyrene, for PAH contaminants. The complete data for a sample taken from the northern margin of the Sado Estuary is presented in Annex A [17]. The spatial distribution of endocrine-disrupting chemical also revealed a different pattern between the industrial areas and the natural reserve areas, corroborating high anthropogenic contamination of some parts of the estuary. Nevertheless, it is also proposed that the hydrodynamic activity of the estuary is possibly involved in the dilution process of the pollutants [38]. Recent studies focused on the genotoxicity in aquatic species exposed to Sado estuarine sediments. Genotoxic damage was observed in *Solea senegalensis*, a common flatfish found in estuaries that feeds on sandy-muddy bottoms, exposed to PAH and metal contaminated sediments, using erythrocyte nuclear abnormalities and the comet assay on fish peripheral blood [17, 41]. These bioassays also demonstrated that the toxicity of sediment contaminants is influenced by synergistic and antagonistic processes that result from the exposure to a complex contaminant mixture [46]. Studies directed to histopathological biomarkers also showed induced hepatic lesions in fish exposed to these contaminated sediments [47, 48].

In summary, contamination due to anthropogenic sources was observed, with a particular distribution along the Sado Estuary. Also, sediment contaminants were revealed to induce genotoxicity in local aquatic species with commercial value. This suggests the need to assess the environmental risk, ecological and to human health, associated with the Sado Estuary sediment contamination, as the retained contaminants can be hazardous to the local benthic populations and to humans exposed through agricultural, recreational or fishing activities, and, more importantly, trough the food chain.

3. Evaluation of sediment contaminants' cytotoxicity and genotoxicity

The exposure to toxic substances can be directly linked to cell survival through its cytotoxic effects, but it can also be linked to mutagenesis and carcinogenesis. The study of toxicity at the genetic level, such as the formation of DNA adducts or chromosomal alterations, as well as DNA damage repair, in experimental cells or animal models, is important in order to characterize agents for their potential to cause genetic damage. Different endpoints can be chosen when evaluating the genotoxic potential, as a first step to understand the risk associated with the exposure to environmental entities. Several methods, *in vitro* or *in vivo*, aiming at analyzing different biomarkers of exposure or effect can be used to evaluate the genotoxic potential of a given xenobiotic or xenobiotic mixture. Presently, among the biomarkers of an early biological effect, the most applied assays are the micronucleus assay and the single-cell gel electrophoresis (or comet) assay. The biomarkers of exposure include the quantification of DNA adducts and the sister chromatid exchange assay, among others. Since many xenobiotics can be genotoxic or produce genotoxic metabolites, the liver is a preferred organ for studying these effects, for it is the key organ in the biotransformation of xenobiotics [1, 49].

3.1. The HepG2 Cell Line

The HepG2 cell line was isolated from a well differentiated hepatocellular carcinoma of a 15 year old Caucasian male. These cells possess certain phase I enzymes, involved in the metabolism of genotoxic carcinogens, as well as phase II enzymes [50]. The HepG2 cells are relatively easy to manipulate and appear to be a practical alternative for assessing genotoxicity because many genotoxic compounds are indirect mutagens [51, 52]. Previous studies showed that the activities of the xenobiotic drug metabolizing enzymes, in these cells, are similar to those observed in freshly isolated primary human hepatocytes, and that these phase I and phase II enzymes retain their activities involved in the biotransformation of mutagens [51]. These properties are of particular interest considering that many of the contaminants present in estuarine sediments, particularly PAHs, require metabolic activation in order to induce a genotoxic effect [20, 21, 30], and that, as previously mentioned, PCB and DDT metabolites can also induce genotoxic damage [30, 32].

3.2. Cytotoxicity Assays: The Neutral Red Uptake Assay

Cytotoxicity assays are widely used in *in vitro* toxicology studies for the detection of cell viability following the exposure to toxic substances. The most commonly used cytotoxicity assays are the lactate dehydrogenase (LDH) leakage assay, the methyl tetrazolium (MTT) assay, the neutral red (NR) uptake assay and the Coomassie protein assay [53]. The NR uptake assay was developed as a cytotoxicity assay based on cell viability chemosensitivity, and is one of the most used for biomedical and environmental applications [54]. Its principle resides on the ability of viable cells to incorporate and bind the supravital dye NR. This dye penetrates the cell's membrane by nonionic passive diffusion and concentrates into the lysosomes. Once in the lysosomes, the dye becomes charged and it is retained inside, *i.e.*, the uptake depends on the cell's capacity to maintain pH gradients. When cell death occurs or the pH gradient is reduced the dye is released out of the cell. Therefore, the amount of retained dye, measured by a colorimetric method, is proportional to the number of viable cells. The NR uptake assay is very sensitive, readily quantifiable and cheap, presents less interference and does not use unstable reagents comparatively to other viability tests [55].

3.3. Genotoxicity Assays

3.3.1. The Alkaline Comet Assay

The single-cell gel electrophoresis, or alkaline comet assay, is a microgel electrophoresis technique for detecting DNA damage at the level of the single cell. To achieve this, cells are embedded in agarose, placed onto a microscope slide, lysed, and the remaining DNA is electrophoresed under alkaline conditions (pH > 13). During the electrophoresis DNA breaks will display an increased migration towards the anode, which combined with the intact nucleoid, can be viewed as "comets" on a fluorescence microscope after staining with ethidium bromide (Figure 3). The level of DNA damage can be measured through the intensity of fluorescence in the comet's tail. In neutral conditions double strand breaks (DSB) can be observed, but at pH > 13, an increase in DNA migration can be associated with increased levels of single strand breaks (SSB) and alkali-labile sites, thus making the alkaline version of the comet assay more sensitive for indentifying genotoxic effects [56]. To further increase the sensitivity of the assay an extra step can be added which involves the incubation of the nucleoids with

an enzyme that recognizes a particular kind of damage and creates a break. Thus different enzymes can be used for different endpoints, allowing the detection of oxidized bases, alkylation damage and UV-induced pyrimidine dimmers. In this way, enzyme-sensitive sites can be converted to additional DNA breaks that can be visualized in the comet assay [57]. The bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (FPG) can be used to detect the major purine oxidation product 8-oxoguanine, as well as other altered purines, reflecting the level of oxidative DNA damage [58]. The comet assay can be an advantageous technique because it is sensitive for the detection of DNA damage, it requires a small number of cells per sample, it is flexible, cheap, relatively fast and it can be conducted with a relatively small amount of test substance [56]. Given the characteristics mentioned above, this method has been widely employed in laboratory genotoxicity and DNA repair studies, as well as in clinical, environmental and human monitoring studies [59].

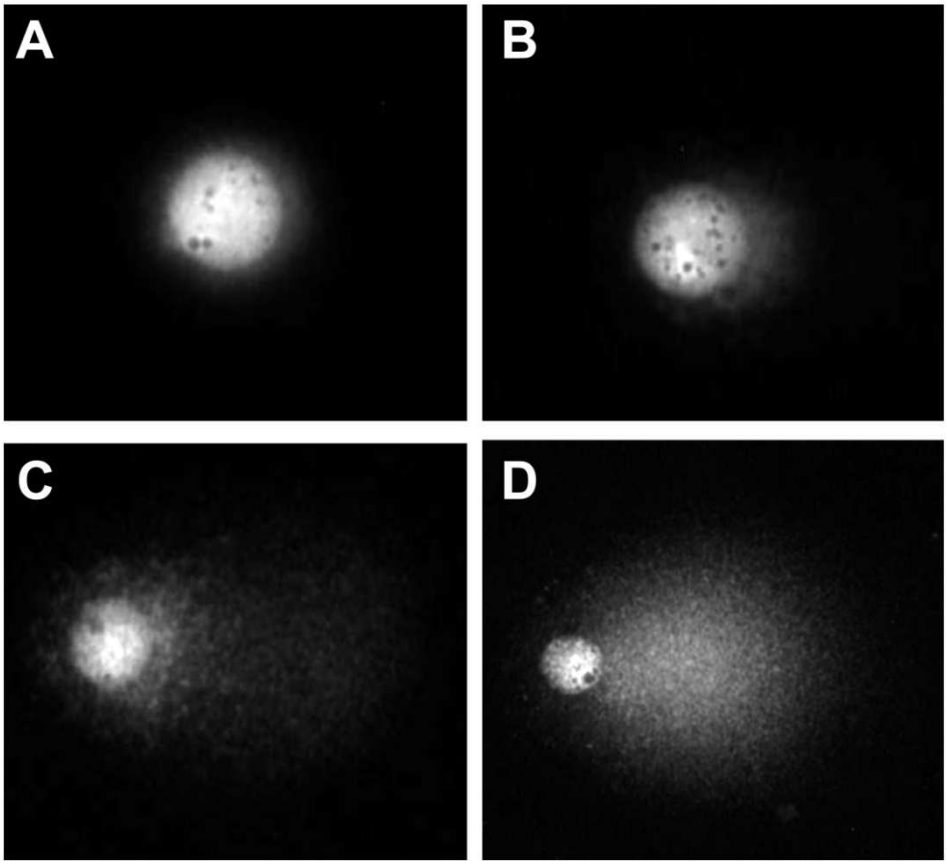

Figure 3 – HepG2 cell nucleoids presenting different levels of DNA damage, observed under a fluorescence microscope (alkaline version of the comet assay). A – no DNA damage; B – low DNA damage; C – moderate DNA damage; D – high DNA damage.

3.3.2. The Micronucleus Assay

Chromosome abnormalities resulting from damage at the DNA and chromosomal level, and chromosome loss or malsegregation – caused by defects in the spindle, centromere or as consequence of undercondensation of the chromosome structure before metaphase – are both important events in cancer [60]. Micronuclei (MNi) originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division, are enveloped at telophase and assume the morphology of an interphase nucleus with the exception of being smaller (Figures 4 and 5). The cytokinesis-block micronucleus (CBMN) assay is the method of choice for quantifying micronuclei *in vitro* or *in vivo*, by scoring them in binucleated cells. In this assay, cells gain their binucleated appearance after blocking the cytokinesis with cytochalasin-B, an inhibitor of microfilament ring assembly required for the completion of cytokinesis (Figures 5A, 5B) [60, 61].

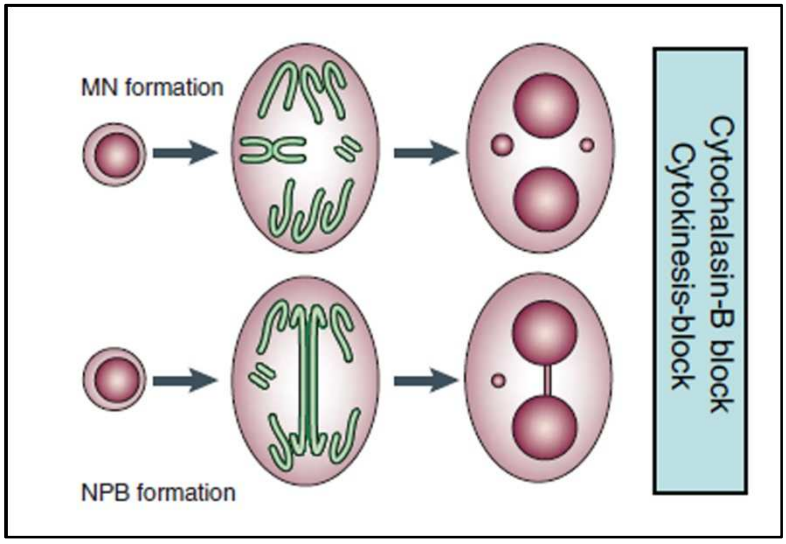

Figure 4 – Micronucleus (MN) and nucleoplasmic bridge (NPB) formation in cells undergoing nuclear division [61].

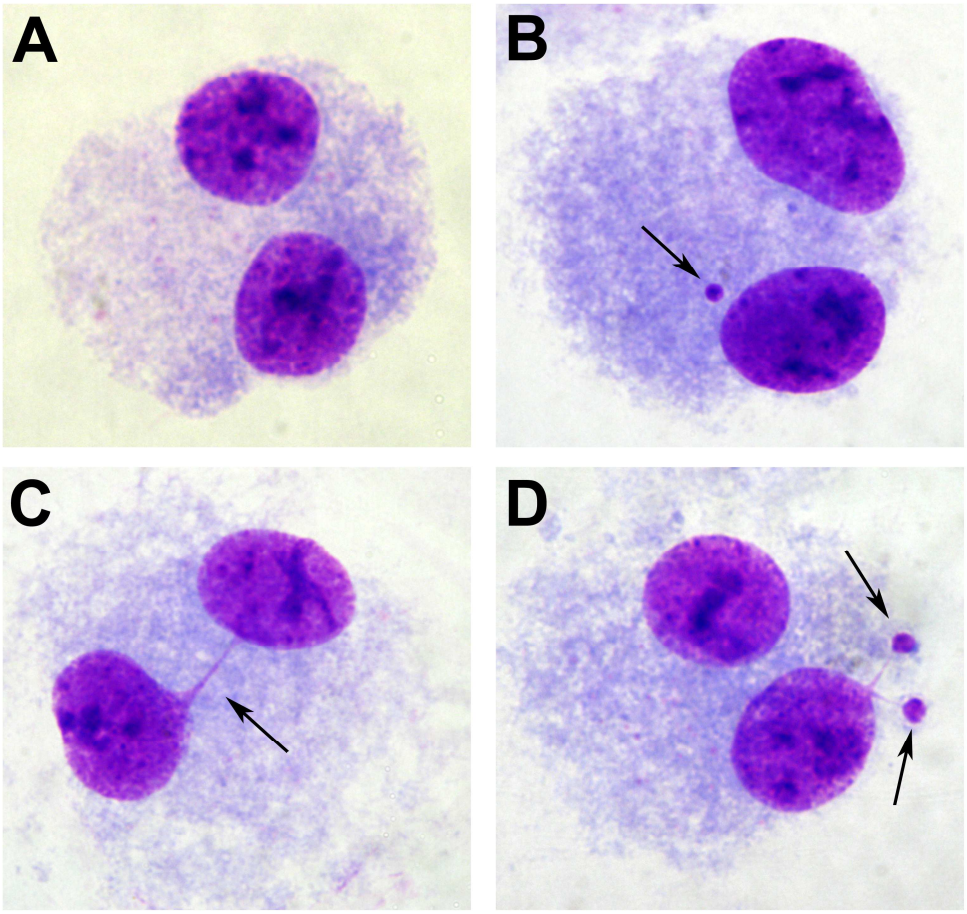

Figure 5 – HepG2 cells observed in the micronucleus assay (bright-field microscopy). **A** – Binucleated HepG2 cell. **B** – HepG2 cell exhibiting one micronucleus. **C** – HepG2 cell exhibiting a nucleoplasmic bridge. **D** – HepG2 cell exhibiting two nuclear buds.

In addition to MNi, this method can be used to measure different endpoints associated with chromosomal damage or instability (Figure 6). Nucleoplasmic bridges are a continuous link between both nuclei, in binucleated cells, and reflect the presence of dicentric chromosomes resulting from telomere end-fusions or DNA misrepair (Figure 5C). Nuclear buds are characterized as being morphologically similar to the micronuclei with the exception of being linked to the nucleus (Figure 5D), and can be scored as an indication of gene amplification [61, 62]. The CBMN assay also allows the measurement of necrotic and apoptotic cells. Therefore, depending on the chosen endpoint, the CBMN assay can be used to analyze DNA damage and misrepair, chromosomal instability, mitotic abnormalities, cell death and cytostasis, enabling direct or indirect measurement of cellular and nuclear dysfunctions. It has become a standard cytogenetic test for genetic toxicology due to its reliability and reproducibility [61].

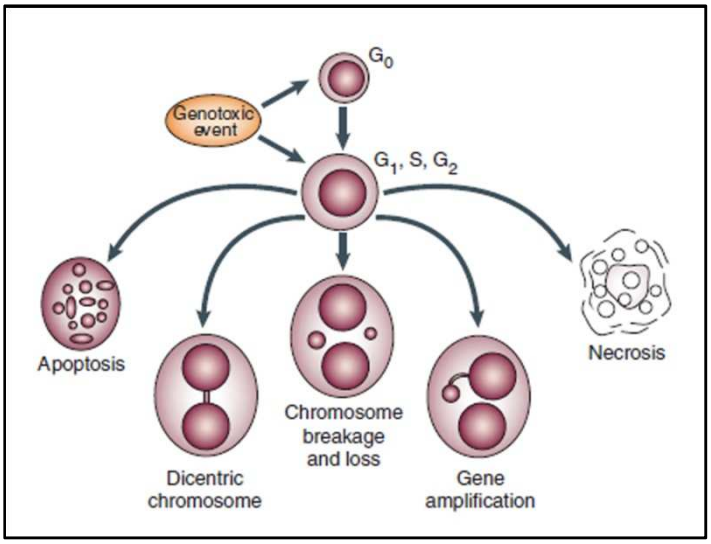

Figure 6 – Various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents. Using the different biomarkers within the CBMN assay, it is possible to establish different endpoints [61].

OBJECTIVES

A growing concern has been directed to the assessment sediment contamination, and its potential risk to aquatic biota and to human population. Previous studies on the Sado Estuary revealed sizable amounts of contaminants in the estuary sediments. These compounds can be accumulated in the edible parts of estuarine species or in agricultural products produced locally, and enter the human food chain, posing a public health problem. The present study is part of a broader project aiming at evaluating the environmental risk, including ecologic and human health risk, associated with the estuarine benthic environment, complemented with the analysis of a target population from a small village located near the southern estuary margin. The general objective of the present study is to preliminarily assess the cytotoxic and genotoxic risk to human health, from exposure to potentially contaminated estuarine sediments, from the Sado Estuary. Thus, using a human liver-derived cell line, the specific objectives of this study are:

- to characterize the Sado Estuary sediments for their cytotoxic potential, through the NR uptake assay;
- to characterize the Sado Estuary sediments for their genotoxic potential, in terms of DNA damage (including oxidative damage), and chromosomal damage, through the alkaline comet assay and the micronucleus assay, respectively.

MATERIAL AND METHODS

1. Sediment sampling

Sediment samples were collected from 4 distinct fishing sites of the Sado Estuary (samples C, A, E and P, Figure 7) and 2 distinct sites of the Mira Estuary (samples M and Mf Figure 8) from late Fall 2010 to Spring 2011 (Table 7). The Mira Estuary (W Portugal) was chosen as a reference estuary for this study, for the previously mentioned reasons. A previously characterized sample (F), collected in May 2007 from a different site in the Sado Estuary, known to induce genotoxicity, was added to serve as a positive control (Annex A) [17]. The sediment samples were collected in permanently submersed sites, homogenized and transported to the laboratory at 4ºC in the dark. Once in the laboratory a portion of the sediment samples was preserved at 4ºC for analysis and another portion was frozen for further studies.

Table 7 – Sediment sampling period, location and environmental characterization.

Estuary	Sample	Sample Sampling Site Characteristics	Sampling Period
Sado	P	Near heavy industrialized area Essentially muddy High hydrodynamics	June 2011
	F	Near the City of Setubal harbor, with influence from heavy industry and urbanism Essentially muddy Low hydrodynamics (Higher water retention)	May 2007
	A	Near area of intense agriculture activities (Mainly rice fields) Fishing grounds Muddy – sandy Low hydrodynamics	May 2011
	E	Area of moderate agriculture activities Fishing grounds Muddy – sandy Low hydrodynamics	May 2011
	C	Farthest from contaminated hotspots (potentially clean) Fishing grounds Essentially sandy Higher hydrodynamics	October 2010
Mira	M	Near an aquaculture area Essentially muddy Low hydrodynamics	January 2010
	Mf	Reference station Essentially sandy	June 2011

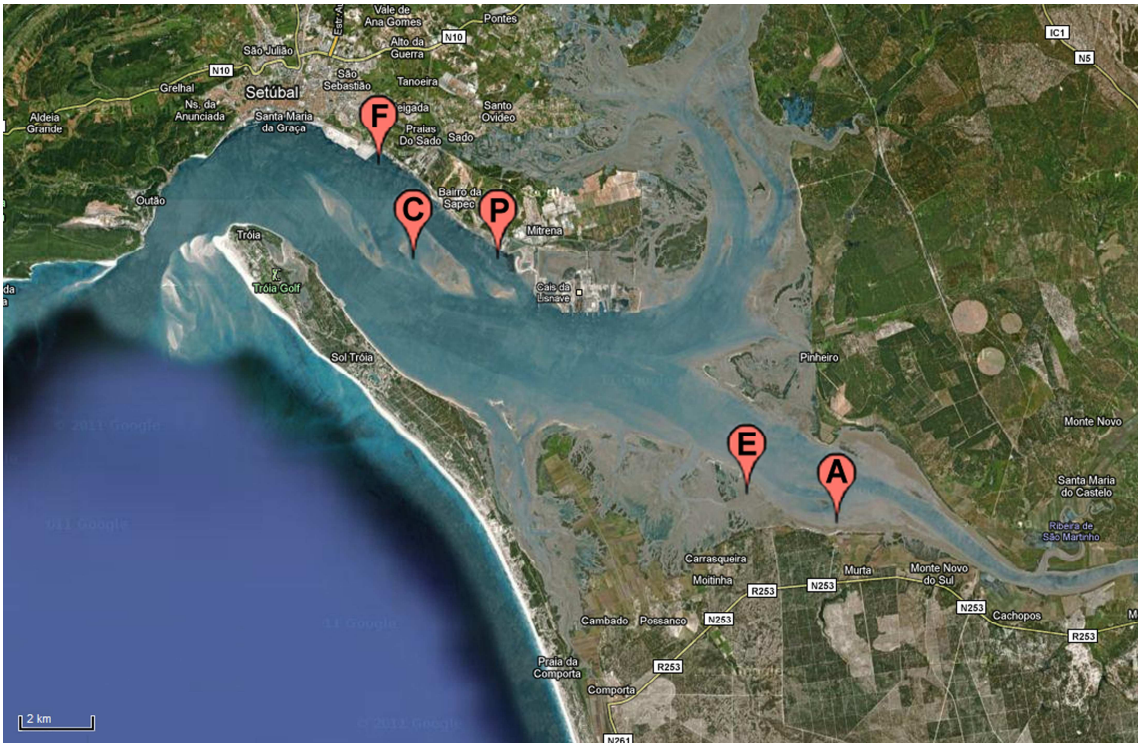

Figure 7 – Satellite map of the Sado Estuary. 📍 indicates the different collection sites (F, P, C, E and A) used in this work (Adapted from [40]).

Figure 8 – Satellite map of the Mira Estuary. 📍 indicates the different collection sites (M and Mf) used in this work (Adapted from [40]).

2. Sediment extracts preparation

Contaminants extraction was adapted from Šrut *et al.* (2011) [12]. Each sediment sample was homogenized and a sub-sample was removed for extraction. Samples were dried for 1 week at 40ºC prior to contaminant extraction, grinded, and 30g of dry sediment was weighed. Reagents used were obtained from Sigma-Aldrich (St. Louis, MO). A mixture of dicloromethane:methanol (2:1) was used to extract a broad range of organic and inorganic contaminants. Extraction was mechanical, for 15min, to ensure maximum contaminant extraction, and allowed to settle in order to obtain two phases (sediment deposit and contaminant extract). The supernatant was removed and the extraction solution was evaporated in a heated plate, at 45ºC. Total organic and inorganic contaminants were then recovered in 3ml of DMSO for cytotoxic and genotoxic assays in cell cultures.

3. Cell culture

The human liver hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC No. HB-8065). All solutions for cell culture were obtained from Invitrogen (Carlsbad, CA). Cells were sub-cultured weekly in DMEM-F12 containing L-Glutamax and HEPES buffer (25 mM), supplemented with 15% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin (10000U penicillin/10 µg streptomycin) and 1,5% amphotericin B (0.25 mg/mL), at 37ºC, in 5% CO_2, humidified atmosphere. The cells were harvested twice a week at 80% confluence using trypsin-EDTA (0.5%).

4. Neutral Red Uptake Assay

HepG2 cells were seeded in 96-well plates at 1×10^4 cells/well and maintained for 24h at 37ºC, in 5% CO_2. Cells were then exposed for 2 and 4h to several concentrations of sediment extracts M, F and C, ranging from 0.1 up to 20µl/ml of culture medium, and for 48h to concentrations of each sediment extract within the same range. Solvent controls were included in all experiments consisting of DMSO at concentrations also ranging from 0.1 up to 20 µl/ml. A negative control was also added consisting of culture medium. All treatment solutions were prepared in culture medium DMEM-F12 with 2% heat-inactivated FBS. For each experiment, 3 replicate wells were used for each treatment condition.

NR assay was carried out according to Repetto *et al.* (2008) [55] with modifications (basic steps are summarized in Figure 9). After cells exposure, treatment medium was removed and replaced with pre-heated (37ºC) fresh growth medium, and NR solution (NR, Merck, Darmstadt, Germany) was added at a final concentration of 50µg/ml. Cells underwent a 3h incubation period, at 37ºC, in 5% CO_2, to allow NR incorporation. After incubation, medium containing NR solution was removed and cells were rinsed twice with pre-heated phosphate buffer saline (PBS). NR/Stop solution (ethanol:acetic acid:water (50:49:1)) was then added to extract the NR dye incorporated in viable cells. After 20min under agitation, the quantity of NR present in the ethanolic solution was measured spectrophotometrically at 540nm using a Multiskan Ascent spectrophotometer (Thermo Labsystems).

Assuming that the mean absorbance of the negative control corresponds to 100% cell viability, relative cell viability of treated cells was calculated by the formula:

$$\text{Relative cell viability (\%)} = \frac{\text{Mean Abs}_{540}\,[\text{treated cells}]}{\text{Mean Abs}_{540}\,[\text{negative control}]} \times 100 \qquad \text{(equation 1)}$$

The results were expressed as the mean value (± SE) of 3 independent experiments per treatment condition.

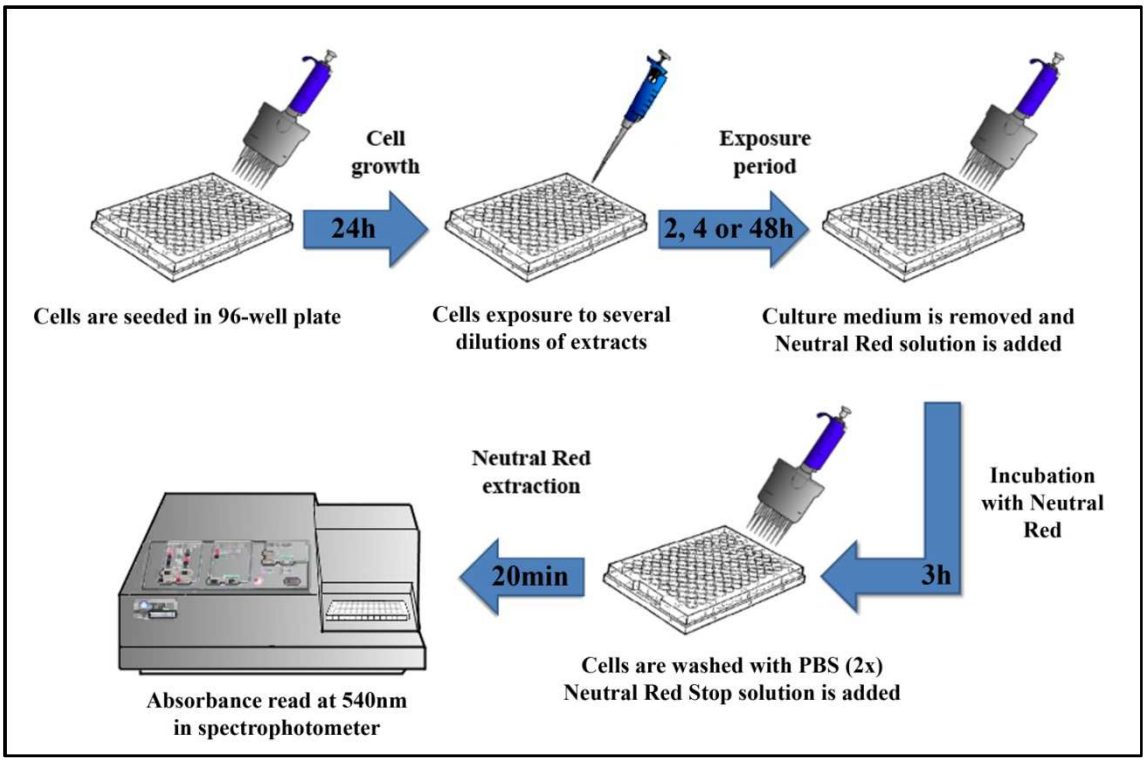

Figure 9 – Basic steps of the neutral red uptake assay.

5. Alkaline Comet Assay with DNA repair endonuclease FPG

All reagents used were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO). Lysis buffer was prepared with 1% of N-Lauroylsarcosine sodium salt; 14.6% of NaCl 2.5M; 3.7% of Na_2EDTA 10mM; 0.12% of Tris-HCl 10mM; 0.8% of NaOH. Lysis solution was freshly prepared for each comet assay with 10% DMSO, 1% Triton-X and 89% of lysis buffer. Electrophoresis buffer was prepared with 1.2% NaOH 300mM; and 0.037% $Na_2EDTA.2H_2O$ 1mM; and pH was ascertained above 13. Neutralization solution was prepared with 4.85% Triz base (0.4 M Tris) and 9.5% HCl 4M. Enzyme buffer F was prepared with HEPES 40mM, KCl 100mM, EDTA acid 0.5mM and BSA 0.2mg/ml. pH was ascertained at 8 with KOH 1M.

HepG2 cells were seeded for 24h in 24-well plates at a density of 5×10^4 cells/well, at 37ºC, 5% CO_2. Cells were then exposed to different concentrations of extract samples, final concentrations from 1 up to 20µl/ml of culture medium, for 48h. A positive control was included in all experiments consisting of H_2O_2 at 200µM, 30min exposure. All treatment solutions were prepared in culture medium DMEM-F12 with 2% heat-inactivated FBS.

The comet assay was then carried out according to Dusinska *et al.* (1996) [63] with modifications (basic steps are summarized in Figure 10). After 48h exposure, cells from each well were rinsed with pre-heated PBS, detached using trypsin-EDTA (0.5%), transferred into eppendorfs and then centrifuged at 1200 rpm, 4ºC, for 10 min. The supernatant was discharged and the cell suspension was embedded in low melting point (LMP) agarose (1%). Two equally distributed drops were then spread onto a microscope slide, previously covered with a 1% normal melting point (NMP) agarose, and covered with a coverslip.

After solidification in a chilled plate, coverslips were carefully removed and the microscope slides were immersed into aluminum foil covered coplin jars (to prevent light induced damage) containing the lysis solution for 1h, at 4ºC. After lysis, slides were rinsed in neutralization buffer and washed 3 times in enzyme buffer F for 5min. FPG (kindly provided by Dr. A. R. Collins, University of Olso, Norway) or enzyme buffer only, was added to each microgel and slides were incubated in metal boxes at 37ºC for 30min. DNA was then allowed to unwind for 40 min in electrophoresis buffer, at 4ºC. Electrophoresis was run for 30 min at 25V and 300mA, at 4ºC. Slides were then rinsed for 10min in neutralization buffer and 10min in distilled H_2O, at 4ºC, and

allowed to dry at room temperature overnight before staining with ethidium bromide (0.125µg/µL).

For each treatment, 100 randomly selected nucleoids (50 from each slide microgel) were analyzed using a fluorescence microscope (Axioplan2 Imaging, Zeiss) equipped with high resolution camera (Zeiss), and using the image-analysis software Comet Imager 2.2 (MetaSystems, GmbH). For each slide the mean percentage of DNA in tail, tail length and tail moment values were obtained. The results were expressed as the mean value (± SE) of 3 independent experiments per treatment condition. Oxidative damage was calculated by subtracting the mean percentage of DNA in tail from untreated nucleoids from the mean percentage of DNA in tail from FPG-treated nucleoids.

In a preliminary set of experiments involving extract samples F, C and M, in concentrations ranging from 0.5 up to 5µl/ml, the alkaline comet assay was carried out uniquely without enzyme treatment, according to Singh *et al.* (1988) [64] with slight modifications. Experimental conditions differed from the above described method in the percentage of LMP agarose used (0.7%), and in that DNA unwinding and electrophoresis were carried out for 20min each; the positive control was EMS at 30mM, 1h exposure.

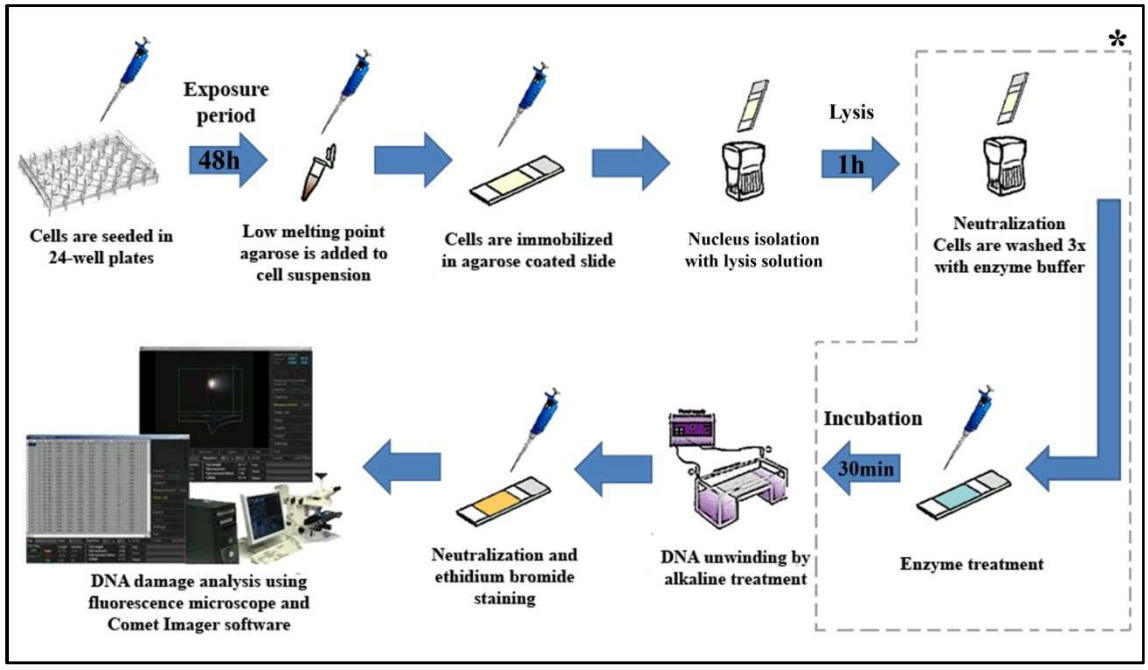

Figure 10 – Basic steps of the alkaline comet assay. *enzymatic treatment steps.

6. Micronucleus Assay

All reagents used were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO). The micronucleus assay was carried out according to M. Fenech *et al.* (2007) [61] with modifications. HepG2 cells were seeded for 24h in 6-well plates at a density of 5×10^4 cells/well, at 37ºC, 5% CO_2. Cells were then exposed to different concentrations of extract samples in duplicate cultures. Mytomycin C at a concentration of 0.3μg/ml was added as a positive control. All treatment solutions were prepared in culture medium DMEM-F12 with 2% heat-inactivated FBS. After 1h exposure, cytochalasin-B was added at a final concentration of 6μg/ml, and cells were incubated for 23h for a total exposure period of 24h.

Following exposure, cells were rinsed with pre-heated PBS, detached with trypsin-EDTA (0.5%), transferred to centrifuge tubes and centrifuged for 5min at 1200rpm. Cell suspension was then submitted to hypotonic shock with KCl 0.1M added drop-by-drop under slight agitation. Cells were fixed with a mixture of methanol:acetic acid (3:1), and then re-fixed with a mixture of methanol (97%) and acetic acid (3%). After removing the supernatant, two drops of the remaining suspension were added directly onto a microscope slide and air dried. For each culture two slides were prepared.

Slides were stained with Giemsa (4%) in phosphate buffer, pH 6.8, mounted with Entelan and analyzed under a light microscope (Axio Imager.A2, Zeiss).

For each treatment, 2000 binucleated cells were analyzed (500 cells per slide, 1000 cells per culture). Results are expressed as the mean value (±SD) of micronuclei per 1000 binucleated cells. The cytokinesis-block proliferation index (CBPI) was calculated by the formula:

$$CBPI = (M_1 + 2M_2 + 3M_3) / N; \qquad \text{(equation 2)}$$

where M_1-M_3 represents the number of cells with 1, 2 or more micronuclei and N is the total number of viable cells scored.

The replication Index (RI) was calculated by the formula:

$$\frac{[((\text{Nº BNC}) + (2 * \text{Nº MNC}))/\text{Total Nº cells}]_{\text{Treated cultures}}}{[((\text{Nº BNC}) + (2 * \text{Nº MNC}))/\text{Total Nº cells}]_{\text{Control cultures}}} \qquad \text{(equation 3)}$$

(BNC – Binucleated cells; MNC – Multinucleated cells)

7. Statistical Analysis

Statistical analysis was performed using SPSS Statistics 17.0.

The NR assay dose-response curves were determined by regression analysis of the data, and IC_{50} values were calculated or extrapolated through the best fitted dose-response curve equation for each sample and for each exposure condition. Statistical analysis of NR assay data was performed either by a parametric one-way ANOVA followed by the Tuckey *post-hoc* test, or by a non-parametric test followed by Dunnett T3 *post-hoc* test, whether variance homogeneity was observed or not.

Statistical analysis of the comet assay data was performed by the non-parametric Kruskal-Wallis test followed by the Mann-Whitney U-test.

Statistical analysis of the micronucleus assay data was performed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney U-test.

Statistical significance, for all assays, was assumed for $p \leq 0.05$.

RESULTS

1. Evaluation of Cytotoxicity: Neutral Red Uptake Assay

The concentrations of each extract tested in the NR assay were chosen up to 20µl/ml, due to solvent cytotoxic interference above this concentration [50].

Following 2h exposure to several concentrations of sediment extracts M, C and F, different responses were obtained. The results are presented in Figure 11. The mean percentages of viability obtained, at the minimum and maximum concentration tested, for each sample, are presented in Table 8. Solvent control showed no significant decrease in cell viability, in the tested concentration range. No dose-response relationships could be established for samples M and C, indicating the absence of an observable cytotoxic effect (Figures 11B and 11C). For sample F, a dose-response curve was established, and the IC_{50}, estimated at 30.07µl/ml, was extrapolated from the curve equation, indicating a mild cytotoxic activity for this sample (Figure 11A). No statistical significance could be obtained for the cytotoxicity induced by any of the extracts, at any concentration, when compared with the negative control. Nevertheless, in a first approach, the comparison of the mean percentage of cell viability, obtained for the highest concentration tested (20µl/ml), of each sample, allows the categorization of the samples according to the level of induced cell death: F > M > C. The complete experimental data for these conditions is presented in Annex B.

Table 8 – Mean percentage of viability obtained for the minimum and maximum tested concentrations, in the neutral red assay, following 2h exposure to each extract sample.

	Mean percentage of viability (±SE)			
Concentration	Sample F	Sample C	Sample M	DMSO
0.1µl/ml (Min)	106.68 ± 8.77	99.18 ± 7.92	120.02 ± 12.12	107.28 ± 7.52
20µl/ml (Max)	65.97 ± 6.61	107.75 ± 3.47	80.20 ± 9.59	106.85 ± 2.71
IC_{50} (µl/ml)	30.07*	-	-	-

Mean percentage of viability (±SE) was obtained from 3 independent experiments.*IC_{50} extrapolated from the obtained curve equation.

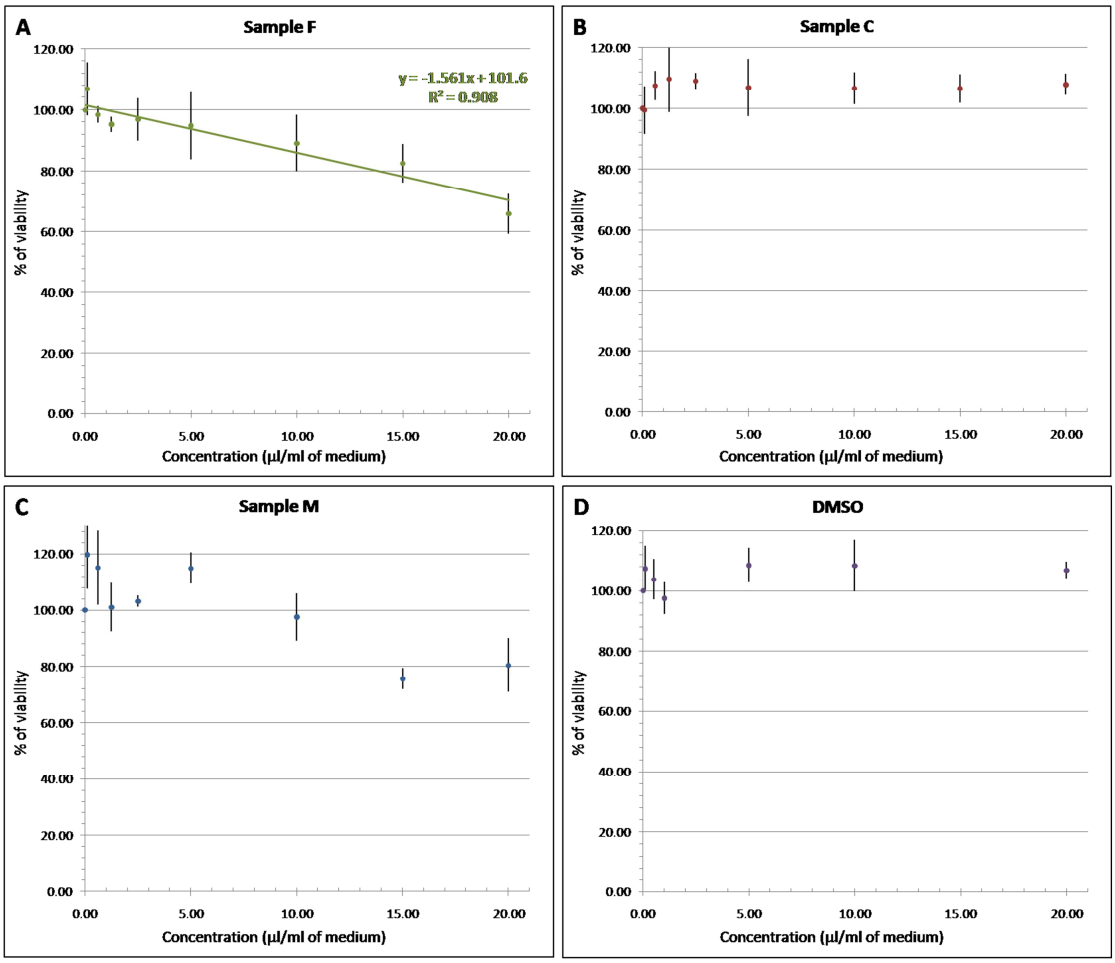

Figure 11 – Neutral red assay results following 2h exposure to different concentrations of the extract samples. Results are expressed as the mean percentage of viability (±SE) of 3 independent experiments. **A** – Sample F; **B** – Sample C; **C** – Sample M; **D** – DMSO.

Following 4h exposure to several concentrations of sediment extracts M, C and F, different responses were also obtained. Results are presented in Figure 12. At minimum and maximum concentrations tested, the mean percentages of viability obtained, for each sample, are presented in Table 9.

Table 9 – Mean percentage of viability obtained for the minimum and maximum tested concentrations, in the neutral red assay, following 4h exposure to each extract sample.

	Mean percentage of viability (±SE)			
Concentration	Sample F	Sample C	Sample M	DMSO
0.1µl/ml (Min)	102.15 ± 11.43	100.78 ± 7.62	111.65 ± 9.19	120.25 ± 10.16
20µl/ml (Max)	48.91 ± 2.89	113.24 ± 7.00	73.41 ± 5.08	100.95 ± 5.50
IC_{50} µl/ml	17.63	-	37.68*	-

Mean percentage of viability (±SE) was obtained from 3 independent experiments. *IC_{50} extrapolated from the obtained curve equation

Treatment with the solvent control showed no significant decrease in cell viability, in the tested concentration range. No dose-response relationship could be established for

sample C, indicating the absence of an observable cytotoxic effect (Figure 12B). Sample M data was best fitted to a linear dose-response curve, and no statistical significance could be obtained for the induced cytotoxicity, at any concentration, when compared with the negative control (Figure 12C). In fact, cell viability at the highest concentration tested does not exhibit a significant decrease, indicating a very weak cytotoxic effect for this sample. For sample F, a linear dose-response curve was established and IC_{50} was calculated at 17.63µl/ml, indicating the presence of a contaminant-driven cytotoxic effect (Figure 12A). Even though IC_{50} for sample F was obtained within the tested concentration range, no statistical significance could be obtained for any of the dilutions tested when compared with the negative control. Nevertheless, when comparing the values of the minimum cell viability obtained at 20µl/ml, samples present again different potential to reduce cell viability (F > M > C). The complete experimental data for these conditions is presented in Annex C.

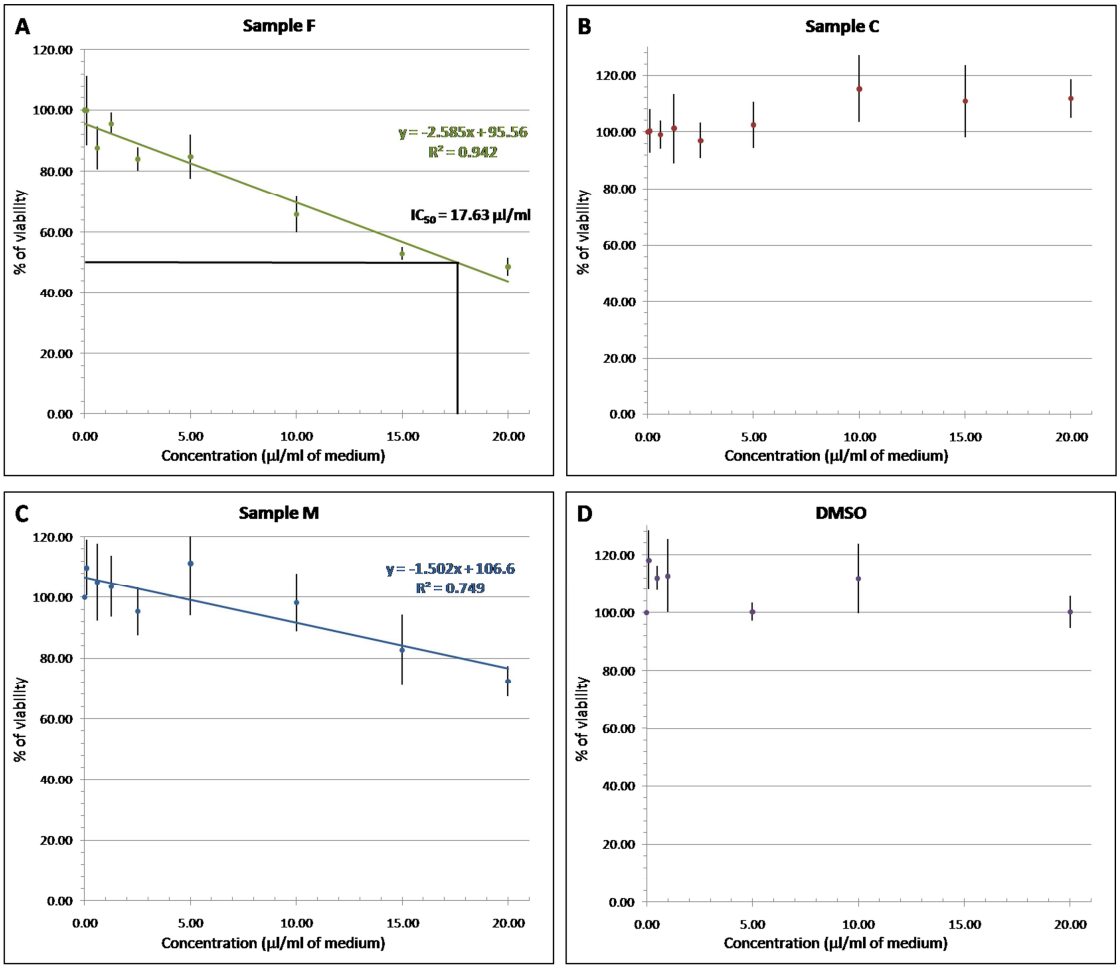

Figure 12 – Neutral red assay results following 4h exposure to different concentrations of extract samples. Results are expressed as the mean percentage of viability (±SE) of 3 independent experiments. **A** – Sample F; **B** – Sample C; **C** – Sample M; **D** – DMSO.

As shown above, following HepG2 cells exposure to extracts M, C and F, during 2 and 4h, weak cytotoxic effects were observed, which led us to consider a longer exposure period of 48h. In addition, extracts from the Sado Estuary samples A, P and E, and from the Mira Estuary sample Mf, were included in the second set of experiments. Following 48h exposures, different cytotoxic patterns were observed for each sample (Figures 13 and 14). The mean percentages of viability obtained, at the minimum and maximum concentration tested, for each sample, are presented in Table 10. A linear-dose response curve was established for solvent control DMSO, but no statistical significance was obtained for any concentration tested when compared with the negative control. Nevertheless, cell viability reduction is observed near the highest concentration tested (67.10% cell viability) indicating, as expected, solvent cytotoxicity beyond this concentration.

Table 10 – Mean percentage of viability obtained for the minimum and maximum tested concentrations, in the neutral red assay, following 48h exposure to each extract sample.

	Mean percentage of viability (±SE)			
Sample	0.1 µl/ml (Min)	20 µl/ml (Max)	*p* value**	IC_{50} (µl/ml)
F	95.06 ± 11.80	9.05 ± 1.24	0.008	5.09
P	101.69 ± 12.77	15.09 ± 3.50	NS	9.24
E	103.87 ± 12.21	45.35 ± 9.47	0.001	19.47
A	108.93 ± 11.92	68.12 ± 12.34	0.047	21.75*
C	105.51 ± 3.81	73.14 ± 5.83	NS	30.38*
M	102.29 ± 3.09	8.80 ± 0.93	0.008	7.31
Mf	104.67 ± 3.16	67.65 ± 7.83	NS	30.79*
DMSO	104.06 ± 5.49	67.36 ± 6.98	NS	-

Mean percentage of viability (±SE) was obtained from 3 independent experiments.*IC_{50} extrapolated from the obtained curve equation.***p* value obtained when statistical significance was observed ($p < 0.05$) for mean percentage of viability, at the highest tested concentration, when compared with negative control. NS – no statistical significance observed.

Samples from the Sado Estuary induced different patterns of cytotoxicity. Results are presented in Figure 13. Samples F and P produced the highest reduction of cell viability up to approximately 91 and 85%, respectively. A dose-response curve was established following a quadratic model for both samples, and IC_{50} were then calculated at 5.09 and 9.24µl/ml, respectively. Statistical significance was obtained for sample F from 11.25 up to 20µl/ml when compared with negative control (with p=0.018 and p=0.008, respectively), and for sample P, no statistical significance could be obtained. However, the use of a parametric approach (ANOVA) shows that statistical significance

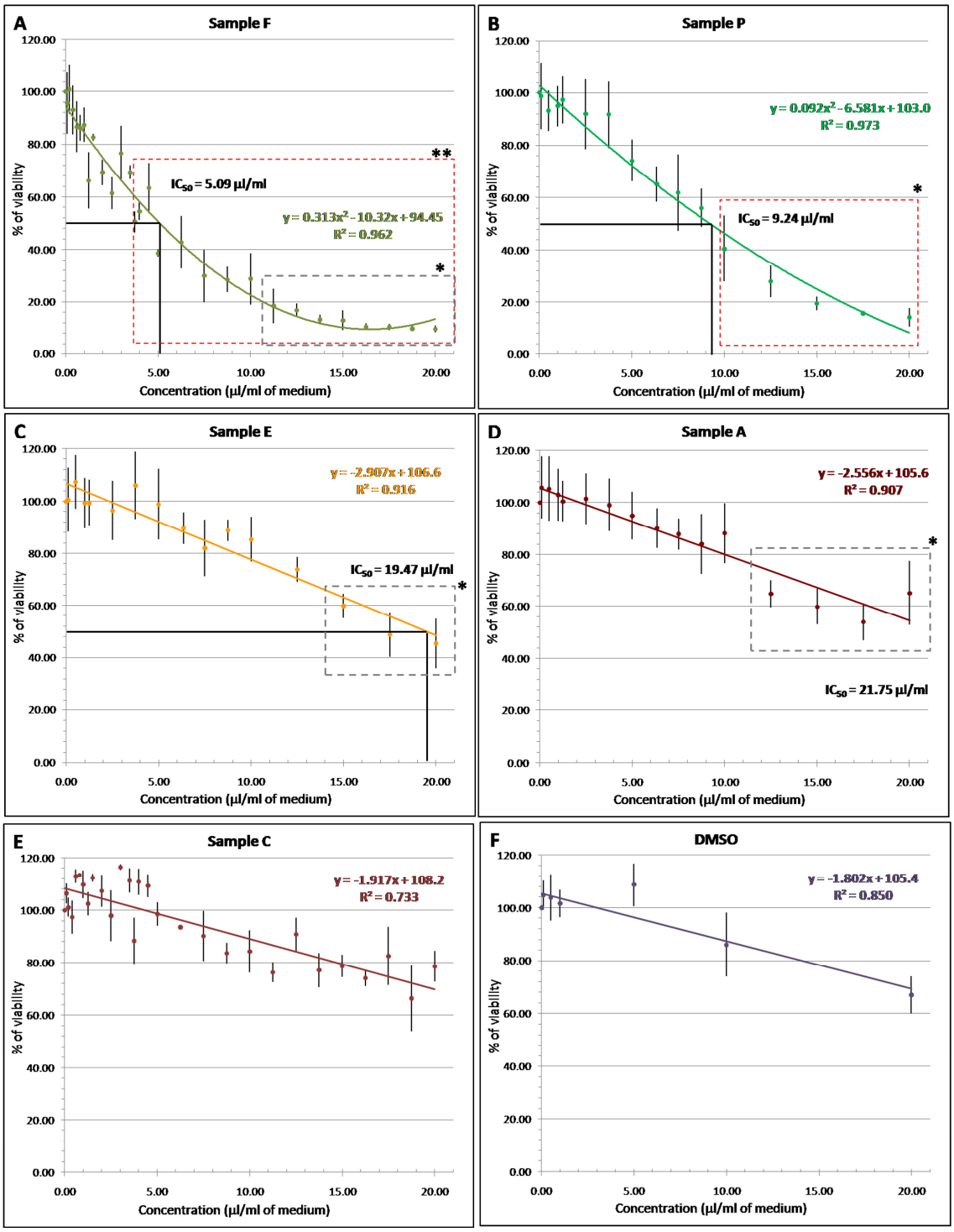

Figure 13 – Neutral red assay results following 48h exposure to different concentrations of extract samples from the Sado Estuary. Results are expressed as the mean percentage of viability (±SE) of 3 independent experiments. **A** – Sample F (*statistical significance, when compared with control, from 11.25 up to 20µl/ml with p=0.018 and p=0.008, respectively.**statistical significance, when compared with control, if a parametric test is used, from 3.75 up to 20µl/ml with p=0.010 and p=0.000, respectively); **B** – Sample P (*statistical significance, when compared with control, if a parametric test is used, from 10 up to 20µl/ml with p=0.036 and p=0.000, respectively); **C** – Sample E (*statistical significance, when compared with control from 15 up to 20µl/ml when compared with negative control, with p=0.026 and p=0.001, respectively); **D** – Sample A (*statistical significance, when compared with control, from 12.5 up to 20µl/ml when compared with negative control, with p=0.044 and p=0.047, respectively); **E** – Sample C; **F** – DMSO.

could be reached from 10 up to 20µl/ml, when compared with the negative control (with p=0.036 and p=0.000, respectively). Likewise for sample F, if a parametric test was used statistical significance would be obtained from 3.75 up to 20µl/ml (with p=0.010 and p=0.000, respectively), which would be more in accordance with the biological meaning of the results. When observing results obtained for these samples, dose-response curves appear to have similar behaviors, but at different concentrations (IC_{50} for sample P was obtained at approximately twice the concentration as that of sample F; Figures 13A and 13B).

Samples A and E produced a reduction of cell viability of approximately 35 and 55%, respectively. The dose-response curves were established following linear models and the IC_{50} were then extrapolated for both samples at 21.75 and 19.47µl/ml, respectively (Figures 13C and 13D). A statistical significant decrease in cell viability was obtained, for sample A, for concentrations from 12.5 up to 20µl/ml (p=0.044 and p=0.047, respectively) and for sample E, for concentrations from 15 up to 20 µl/ml (p=0.026 and p=0.001, respectively), when compared with the negative control. Overall, the dose-response relationship for both samples appears to be similar, but sample E showed clear evidence of contaminant-driven cytotoxicity, attending to its IC_{50} that was obtained within the tested concentration range. Nevertheless, both samples revealed low cytotoxic effects. In fact, sample A results are very similar to the solvent control, even though statistical significance was observed.

Sample C produced a reduction of approximately 20% in cell viability. A dose-response curve was established following a linear model. No statistical significance was obtained for any concentration when compared with the negative control. The IC_{50} for this sample was not extrapolated because the dose-response curve appears to be similar to solvent control at the same dose-level, indicating that it might not produce cytotoxicity in this cell line (Figure 13E).

Samples from the Mira Estuary also displayed different cytotoxic patterns (Figure 14). On the one hand, cell viability reduction was observed up to approximately 90% for sample M. A dose-response curve was established following a quadratic model and the IC_{50} was then calculated at 7.31µl/ml. A statistical significant decrease in cell viability was obtained for dilutions from 11.25µl/ml up to 20µl/ml, when compared with the negative control (p=0.005 and p=0.008, respectively) indicating high cytotoxicity (Figure 14A). On the other hand, sample Mf showed a moderate reduction

of cell viability up to approximately 35%, similar to that observed, for comparable dilutions, with the solvent control. A dose-response curve was established following a linear model. No statistical significance was obtained for any concentration when compared with the negative control. These results indicate either a very low or null cytotoxic effect relatively to the solvent control (Figure 14B).

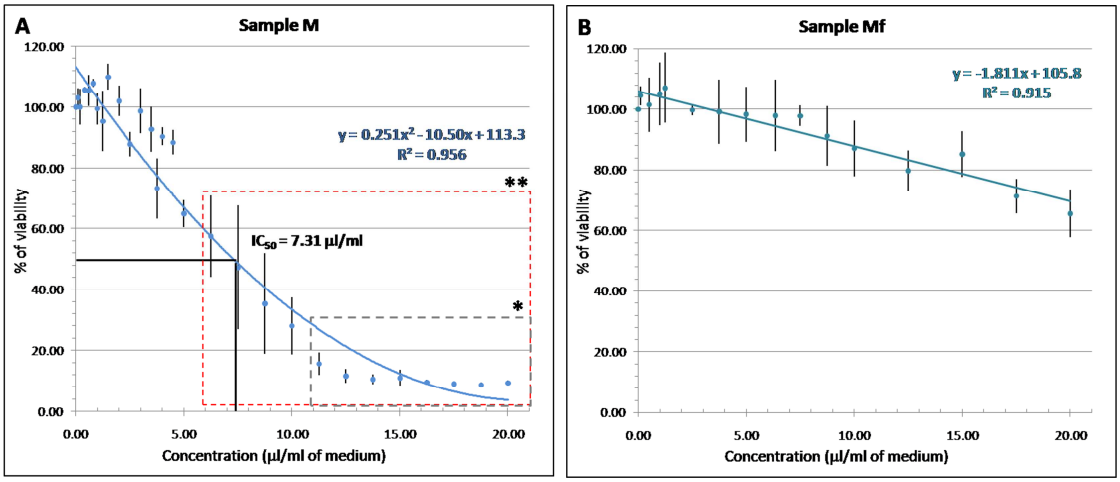

Figure 14 – Neutral red assay results with 48h exposure to different concentrations of extract samples from the Mira Estuary. Results are expressed as the mean percentage of viability (±SE) of 3 independent experiments. **A** – Sample M (*statistical significance, when compared with control, from 11.25 up to 20µl/ml with *p*=0.005 and *p*=0.008, respectively. **statistical significance, when compared with control, if a parametric test is used, from 6.25 up to 20µl/ml with *p*=0.027 and *p*=0.000, respectively); **B** – Sample Mf;

Even though extracts from each sample showed different patterns of induced cytotoxicity in HepG2 cells, samples can be arbitrarily grouped according to their pattern in the following way: samples F, P and M by producing a pronounced reduction in cell viability; sample E by inducing a moderate reduction in cell viability; and samples A, C and Mf as not cytotoxic, within the tested concentration range. Samples can be ranked for their cytotoxicity, based on the calculated (or extrapolated) IC_{50}, as follows: F > M > P > E > A, C, Mf. When compared with the mean percentage of cell viability obtained for the highest concentrations used, sample ranking according to cytotoxicity is similar. The complete experimental data for these conditions is presented in Annex D.

2. Evaluation of Genotoxicity

2.1. Alkaline Comet Assay

In a first approach, analysis of the genotoxic effects of several dilutions of the three available extract samples (C, M and F), through the standard alkaline comet assay, revealed no significant induction of DNA damage. Results are presented in Figure 15, and the complete experimental data for these conditions is presented in Annex E. These preliminary negative results, together with our knowledge that many contaminants, possibly present in the sediment samples, are able to induce DNA damage through oxidative stress, led us to try increasing the sensitivity of the comet assay, and to direct our experiments to a different endpoint. On the one hand the time of DNA unwinding and of electrophoresis was extended and, on the other hand, nuclei were exposed to the FPG enzyme to reveal FPG sensitive sites (oxidized purines) that reflect oxidative DNA damage.

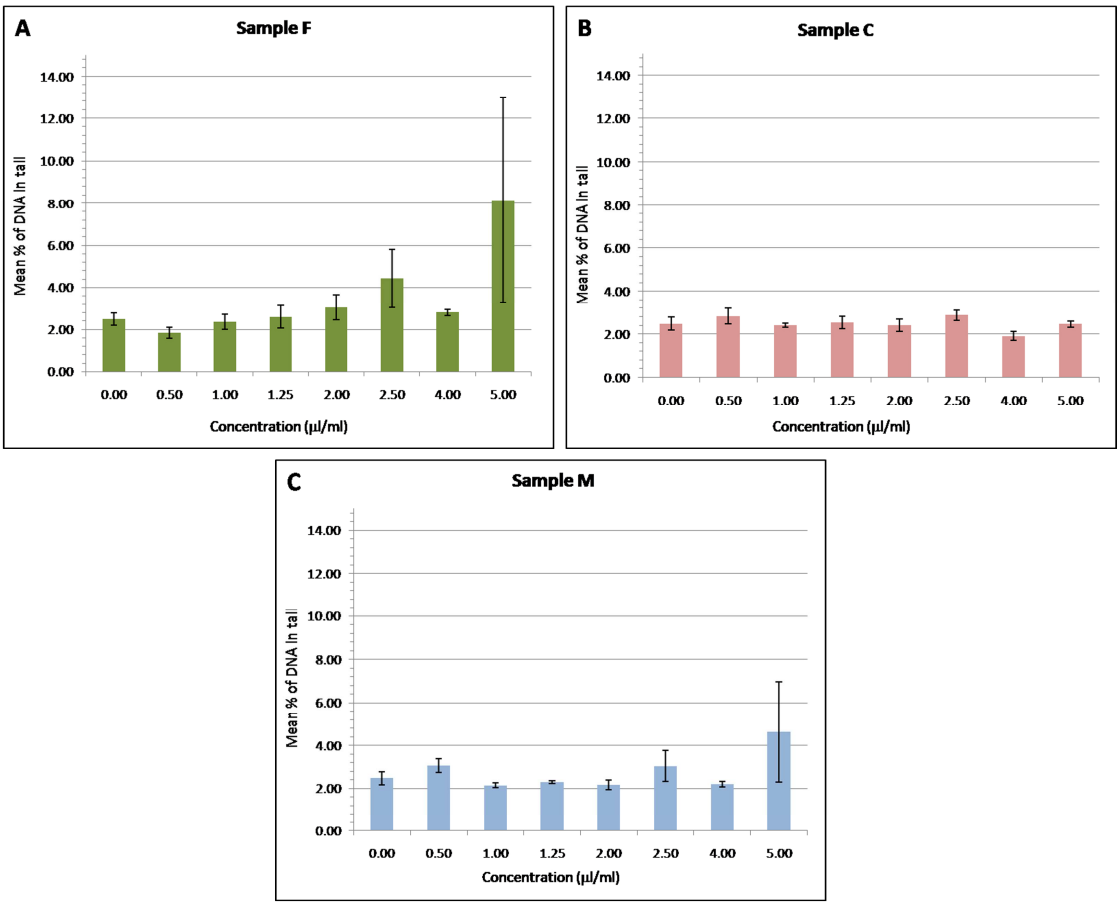

Figure 15 – Results from the standard alkaline comet assay, following 48h exposure to different concentrations of extract samples. Results are expressed as the mean percentage of DNA in tail (± SE) from 3 independent experiments. **A** – Sample F; **B** – Sample C; **C** – Sample M. Negative control refers to DMSO (5µl/ml).

Sediment extracts concentrations to be tested for their genotoxicity by the comet assay, were chosen according to the results obtained in the NR assay (48h exposure period). The top concentration tested was the one that induced 50±5% cytotoxicity. When the IC_{50} concentration was not reached, the top concentration was mostly 20µl/ml, to avoid solvent cytotoxicity interference.

Following modifications of the experimental conditions, after 48h exposure to different concentrations of sediment extracts, HepG2 cells presented different levels of DNA damage, in terms of total DNA strand breakage. All samples, except sample C, showed a concentration-related increase in the mean percentage of DNA in tail, with FPG treatment. In addition, a significant difference in the DNA strand break level was observed between FPG-treated and untreated nuclei, at least for the highest concentrations tested. Results obtained for the Sado Estuary extracts are presented in Figure 16, and complete data for tail length and tail moment are displayed in Annex F (Tables F1 – F5).

In these different experimental conditions, sample F (2.5 and 5µl/ml), used as a positive control, was able to produce a significant increase in DNA damage, when compared with the solvent control (p=0.05), even without FPG treatment, confirming the improvement of the comet assay sensitivity to detect DNA damage. Following nucleoids treatment with FPG, the DNA damage induced by the extract from sample F was significantly raised for all concentrations tested, comparatively to untreated nuclei (p=0.05), suggesting the presence of oxidative lesions in DNA (Figure 16A). Cells exposure to the extract from sample P resulted in a significant increase in the mean percentage of DNA in tail, at concentration of 10µl/ml without treatment with FPG, and at concentrations of 1, 2.5, 5 and 10µl/ml with FPG treatment, when compared with the respective solvent controls (p=0.05). Significant differences between nucleoids treated and untreated with FPG were observed at each tested concentration (Figure 16B). The overall genotoxic pattern observed for both samples F and P appear to be similar, with sample P revealing higher DNA damage induction, both with and without FPG treatment, at the two highest tested concentrations.

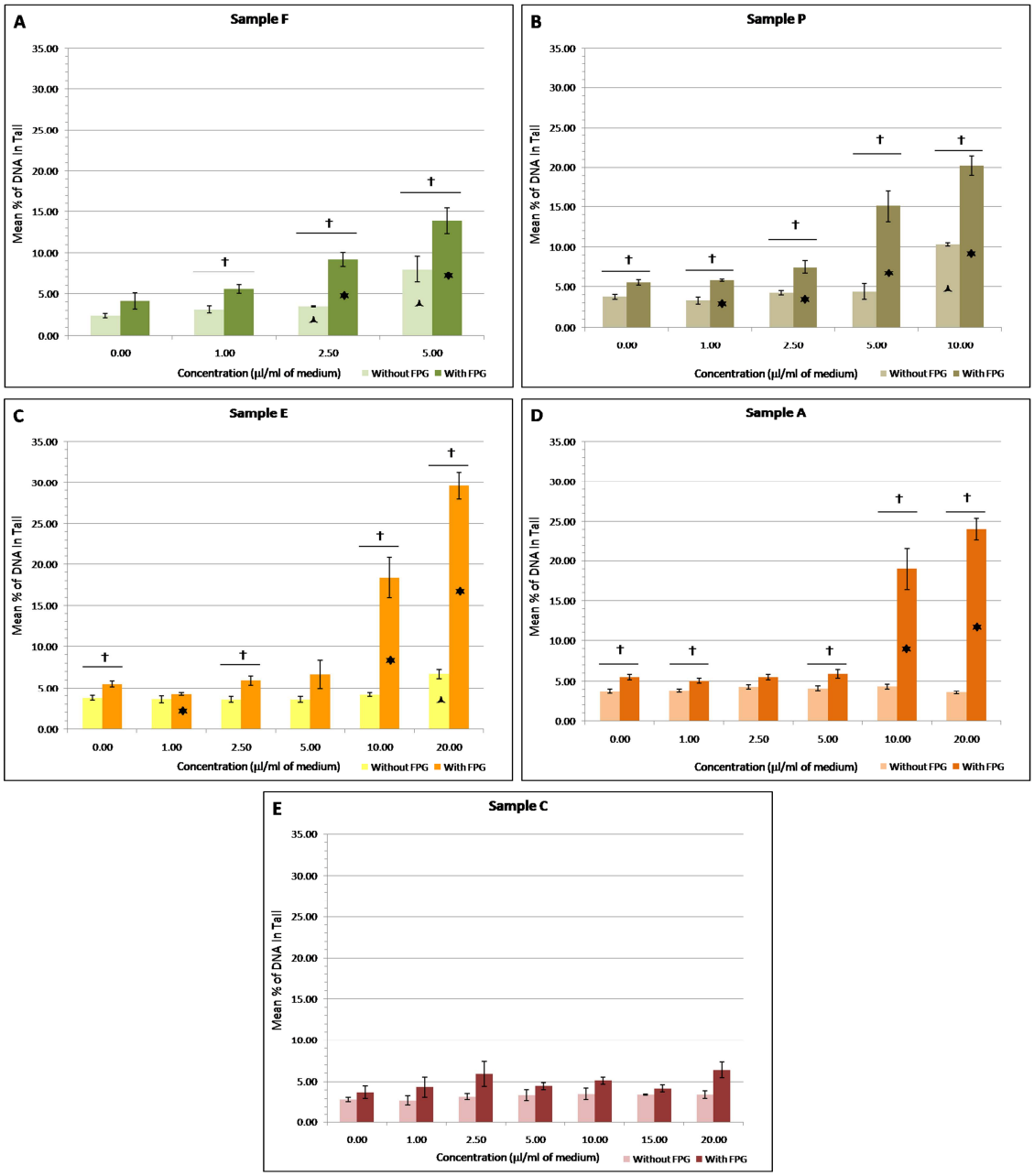

Figure 16 – Alkaline comet assay results following 48h exposure to different concentrations of extract samples from the Sado Estuary. Results are expressed as the mean percentage of DNA in tail (± SE) from 3 independent experiments. **A** – Sample F; **B** – Sample P; **C** – Sample E; **D** – Sample A; **E** Sample C. 0µl/ml corresponds to 0.05% DMSO for sample F, and 2% DMSO for samples P, E, A and C. † – Statistical significant difference between treatment with and without FPG, at the same concentration. ⅄ – Statistical significant difference over the solvent control (without FPG treatment). ✱ – Statistical significant difference over the solvent control (with FPG treatment).

Cells exposure to the sample E extract produced a significant increase in the mean percentage of DNA in tail, without FPG treatment, at the concentration of 20µl/ml, when compared with the solvent control (p=0.05). Following treatment with FPG, the extract concentrations of 10 and 20µl/ml generated a significant increase in the total level of DNA damage, when compared with the respective solvent control

(p=0.05). Also, a significant difference in DNA damage observed, between FPG treated and untreated nucleoids, for concentrations of 2.5, 10 and 20µl/ml (p=0.05), revealed the presence of significant oxidative DNA damage (Figure 16C). Cells exposure to the sample A extract failed to increase DNA damage, for all tested concentrations. Nevertheless, when FPG treatment was included, the same extract originated a significant increase in DNA damage at concentrations of 10 and 20µl/ml. Significant additional oxidative DNA damage was observed for all tested concentrations, except for that of 2.5µl/ml (p=0.05) (Figure 16D). The overall genotoxic pattern observed for samples E and A appear to be similar, with sample E revealing a higher level of induced DNA damage, at the two highest tested concentrations, both with and without FPG treatment.

In contrast, for sample C no significant difference between treatment with and without FPG was observed. In addition, no significant increase in the mean percentage of DNA in tail was observed with increasing concentrations of the extract. Sample C was therefore unable to induce genotoxic damage in the experimental conditions used (Figure 16E).

The two samples from the Mira Estuary also revealed different patterns of genotoxicity after 48h exposure. Results are presented in Figure 17, and data for tail length and tail moment are displayed in Annex F, Tables F6 and F7. Sample M was able to induce a significant increase in the mean percentage of DNA in tail only at the highest concentration tested (5µl/ml), when compared with the solvent control (p=0.05). When treatment with FPG was included, a significant increase in DNA damage was observed at concentrations of 1 and 2.5µ/ml (p=0.05). Significant additional oxidative DNA damage was also observed between treatment with and without FPG at concentrations of 1 and 2.5µl/ml (Figure 17A). The failure to obtain a significant level of oxidative DNA damage at the concentration of 5µl/ml, when compared with the solvent control, might be due to the high dispersion of the results obtained in the three independent experiments, even though the mean percentage of DNA in tail corresponded to 19.29%. As for sample Mf, it did not produce a significant increase in DNA damage, when compared with the solvent control. A significant increase in DNA damage, in the presence of FPG treatment, was only observed at the highest tested concentration (20µl/ml), when compared with the solvent control (p=0.05), although it corresponded to 7.20% of DNA in tail (Figure 17B).

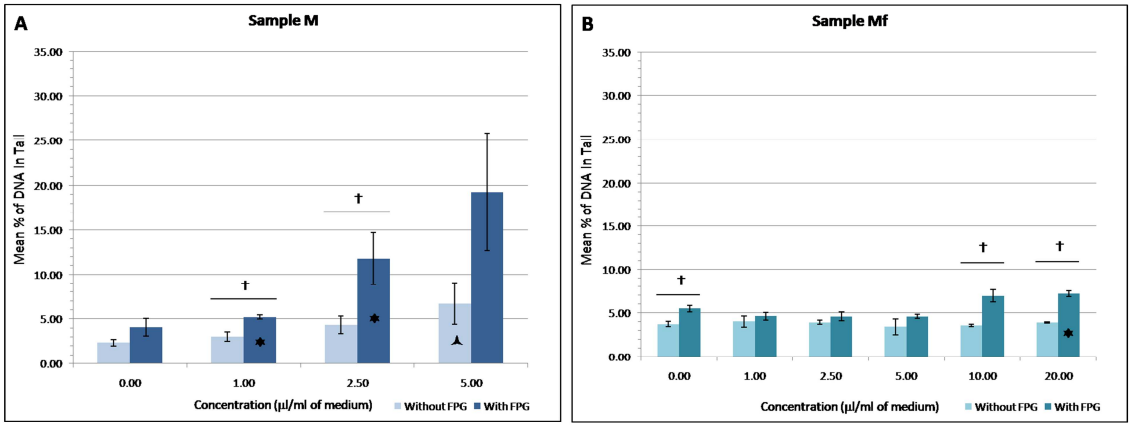

Figure 17 – Alkaline comet assay results following 48h exposure to different concentrations of extract samples from the Mira Estuary. Results are expressed as the mean percentage of DNA in tail (± SE) from 3 independent experiments. **A** – Sample M (concentration 0 refers to DMSO at 5µl/ml); **B** – Sample Mf (concentration 0 refers to DMSO at 20µl/ml). † – Statistical significance between treatment with and without FPG, at the same concentration. ⅄ – Statistical significance when compared with the respective solvent control without FPG treatment. ✱ – Statistical significance when compared with the respective solvent control with FPG treatment.

Considering the highest concentration of each extract tested in the comet assay, and based in the level of induced oxidative DNA damage (FPG sensitive sites; calculated as the [mean % of DNA damage with FPG] – [mean % of DNA damage without FPG]) extracts can be ranked as follows: E > A > M > P > F > Mf > C (Table 11). The results show that extracts E and A are able to produce, approximately two-fold higher levels of oxidative DNA damage, than those from extracts F and P. This genotoxic potency ranking is however different from the one obtained when considering DNA damage without FPG treatment (P > F > E > M > Mf > A > C).

Table 11 – Results of the mean percentage of DNA in tail (with and without FPG treatment), FPG sensitive sites, and sample ranking from lowest to highest DNA damage induction, at the highest concentration tested per sample.

Samples ordered from lowest to higest DNA damage induction ↓	Mean % of DNA damage (±SE)		Mean % of DNA damage with FPG treatment (±SE)		Mean % FPG sensitive sites (±SE)**	
C	3.44 ± 0.47	C	6.37 ± 0.95	C	2.93 ± 1.12
A	3.59 ± 0.19	Mf	7.20 ± 0.35*	Mf	3.26 ± 0.42
Mf	3.94 ± 0.07	F	13.84 ± 1.57*	F	5.87 ± 3.13
M	6.72 ± 2.32*	M	19.24 ± 6.56	P	9.90 ± 1.23*
E	6.72 ± 0.55*	P	20.22 ± 1.20*	M	12.57 ± 4.34*
F	7.98 ± 1.58*	A	24.01 ± 1.33*	A	20.42 ± 1.51*
P	10.32 ± 0.19*	E	29.63 ± 1.66*	E	22.91 ± 1.46*

Results are expressed as the mean (±SE) of 3 independent experiments.*statistically significant when compared with the respective solvent control (p=0.05). **Calculated as [mean % of DNA damage with FPG] – [mean % of DNA damage without FPG].

Results of the comparison of the extracts genotoxicity at equi-toxic concentrations are presented in Tables 12 and 13. In approximate equi-toxic

concentrations, relative to a reduction in cell viability up to 25%, samples P, E, A and M were able to induce significant DNA damage, but uniquely with FPG treatment. In approximate equi-toxic concentrations, relative to a reduction in cell viability from 25 up to 50%, samples F, P, E and M induced significant levels of DNA damage in HepG2 cells (with sample P displaying the highest level), whereas in the presence of FPG, DNA breaks induced by samples A and Mf also become evident (with sample E displaying the highest level). At these approximate equi-toxic concentrations, samples P, E and A, revealed the highest potency to raise the levels of DNA damage, when FPG treatment was included.

Table 12 – Results of the mean percentage of DNA in tail (with and without FPG treatment), and FPG sensitive sites, for each sample at approximate equi-toxic concentrations, inducing up to 25% reduction in cell viability.

Sample	Concentration (µl/ml)**	Mean % of DNA damage (±SE)	Mean % of DNA damage with FPG treatment (±SE)	Mean % FPG sensitive sites (±SE)
F	1	3.10 ± 0.44	5.56 ± 0.50	2.46 ± 0.93
P	5	4.42 ± 0.95	15.10 ± 1.97*	10.69 ± 2.54
E	10	4.16 ± 0.22	18.39 ± 2.41*	14.23 ± 2.60
A	10	4.38 ± 0.29	19.02 ± 2.58*	14.63 ± 2.36
C	15	3.42 ± 0.06	4.17 ± 0.42	0.75 ± 0.47
M	2.5	4.34 ± 0.99	11.78 ± 2.91*	7.45 ± 3.84
Mf	10	3.60 ± 0.13	6.97 ± 0.70	3.37 ± 0.83

Results are expressed as the mean (±SE) of 3 independent experiments.*statistically significant when compared with the respective solvent control (p=0.05). **Concentrations were chosen, through the highest reduction of cell viability, in the approximate range of 100 to 75% of cell viability obtained in the neutral red assay, and taking into account concentrations tested in the comet assay.

Table 13 – Results of the mean percentage of DNA in tail (with and without FPG treatment), FPG sensitive sites, for each sample at approximate equi-toxic concentrations, inducing 25 up to 50% reduction in cell viability.

Sample	Concentration (µl/ml)**	Mean % of DNA damage (±SE)	Mean % of DNA damage with FPG treatment (±SE)	Mean % FPG sensitive sites (±SE)
F	5	7.98 ± 1.58*	13.84 ± 1.57*	5.87 ± 3.13
P	10	10.32 ± 0.19*	20.22 ± 1.20*	9.90 ± 1.23
E	20	6.72 ± 0.55*	29.63 ± 1.66*	22.91 ± 1.46
A	20	3.59 ± 0.19	24.01 ± 1.33*	20.42 ± 1.51
C	20	3.44 ± 0.47	6.37 ± 0.95	2.93 ± 1.12
M	5	6.72 ± 2.32*	19.29 ± 6.56	12.57 ± 4.34
Mf	20	3.94 ± 0.07	7.20 ± 0.35*	3.26 ±0.42

Results are expressed as the mean (±SE) of 3 independent experiments.*statistically significant when compared with the respective solvent control (p=0.05). **Concentrations were chosen, through the highest reduction of cell viability, in the approximate range of 75 to 50% of cell viability obtained in the neutral red assay, and taking into account concentrations tested in the comet assay.

Considering that sample F was included in this study as a positive control, the mean percentage of DNA in tail for all samples, was compared with that of sample F, at the same concentrations used, with or without FPG treatment (Table 14). In general, the level of DNA strand breaks, produced in HepG2 cells by extracts from samples P and M, did not differ significantly from that of sample F, suggesting that the three samples are genotoxic. Comparison of samples, A, E, C and Mf, with the positive control, revealed that the significant differences are mainly observed at the highest concentrations tested of sample F, showing lower genotoxicity for these samples.

Table 14 – Pair wise comparison of each sample with sample F, for the induction of DNA damage at each tested concentration, with and without FPG treatment.

Concentration (µl/ml) for sample F	Samples					
	P	E	A	C	M	Mf
1 (without FPG)	ns	ns	ns	ns	ns	ns
1 (with FPG)	ns	S	ns	ns	ns	ns
2.5 (without FPG)	S	ns	S	ns	ns	ns
2.5 (with FPG)	ns	S	S	ns	ns	S
5 (without FPG)	ns	S	S	S	ns	ns
5 (with FPG)	ns	S	S	S	ns	S

Comparison was performed by the Mann-Whitney U-test. **S** – statistical significant difference between samples (p=0.05). **ns** – No statistical significant difference.

As seen in the NR assay, analysis of the overall comet assay results, uncover similar patterns of induced cytotoxicity and genotoxicity, in some groups of samples. Taking this information into account, as well as their collection site information, a pair wise comparison, for comet assay results, was made between samples with similar genotoxic, cytotoxic and collection site information in the following groups: samples P, F and M; samples E and A; and samples C and Mf. When samples C (potentially clean, Sado) and Mf (reference, Mira) were compared at equal concentrations, no significant statistical difference was observed between them at any concentration with or without FPG treatment. When samples E and A, from the southern margin of the Sado Estuary, were paired, a statistically significant difference was only observed at the concentrations of 2.5 and 20µl/ml without FPG treatment (p=0.05). In turn, samples F and P, from the northern margin of the Sado Estuary, only exhibit significant statistical difference at the concentration of 2.5µl/ml without FPG treatment (p=0.05). Nevertheless, when compared with their equi-toxic effects, samples differ significantly at the concentration

2.5 vs. 5µl/ml with FPG treatment (p=0.05) and 5 vs. 10µl/ml with FPG treatment (at their IC_{50} concentrations, p=0.05). Overall patterns at the same concentration might be similar, but at equi-toxic concentration both samples differ in their induction of oxidative DNA damage. When comparison with sample M was included with both samples F and P, statistical significance could only be observed between sample M and P at concentrations 1 and 2.5µl/ml with FPG treatment (p=0.05).

Nucleoids where arbitrarily classified by their percentage of DNA in tail in five classes, for each tested concentration with FPG treatment, and results are presented in Figure 18. In the absence of FPG treatment (Annex F, Figure F1), only nucleoids from class 0 (0% ≤ DNA in tail < 20%), were measured, except for samples F, P and M, at the highest tested concentration, that revealed a small percentage of nucleoids from classes 1 (20% ≤ DNA in tail < 40%) and 2 (40% ≤ DNA in tail < 60%). Following FPG treatment, categorization of nucleoids in classes yielded results that support those obtained with the measurement of DNA in tail. Nevertheless, when comparing samples F and P, we observed a highest presence of nucleoids from classes 1 and 2 in sample P, at equi-toxic concentrations (two highest concentrations tested), with FPG treatment. As for samples E and A, classification shows that sample E was able to induce approximately twice as much nucleoids from class 2 as sample A, and induce nucleoids from class 3, at the highest concentrations tested, with FPG treatment. So, even though overall nucleoid classification appear to be similar between these two samples, sample E once again reveals a different genotoxic profile from sample A, at the highest concentration tested (20µl/ml). Sample M, in comparison with samples F and P, at the highest tested concentration, exhibits a higher induction of nucleoids from classes 2 and 3, which might suggest a different type of contamination in this sample. Samples C and Mf exhibited rare nucleoids above class 0.

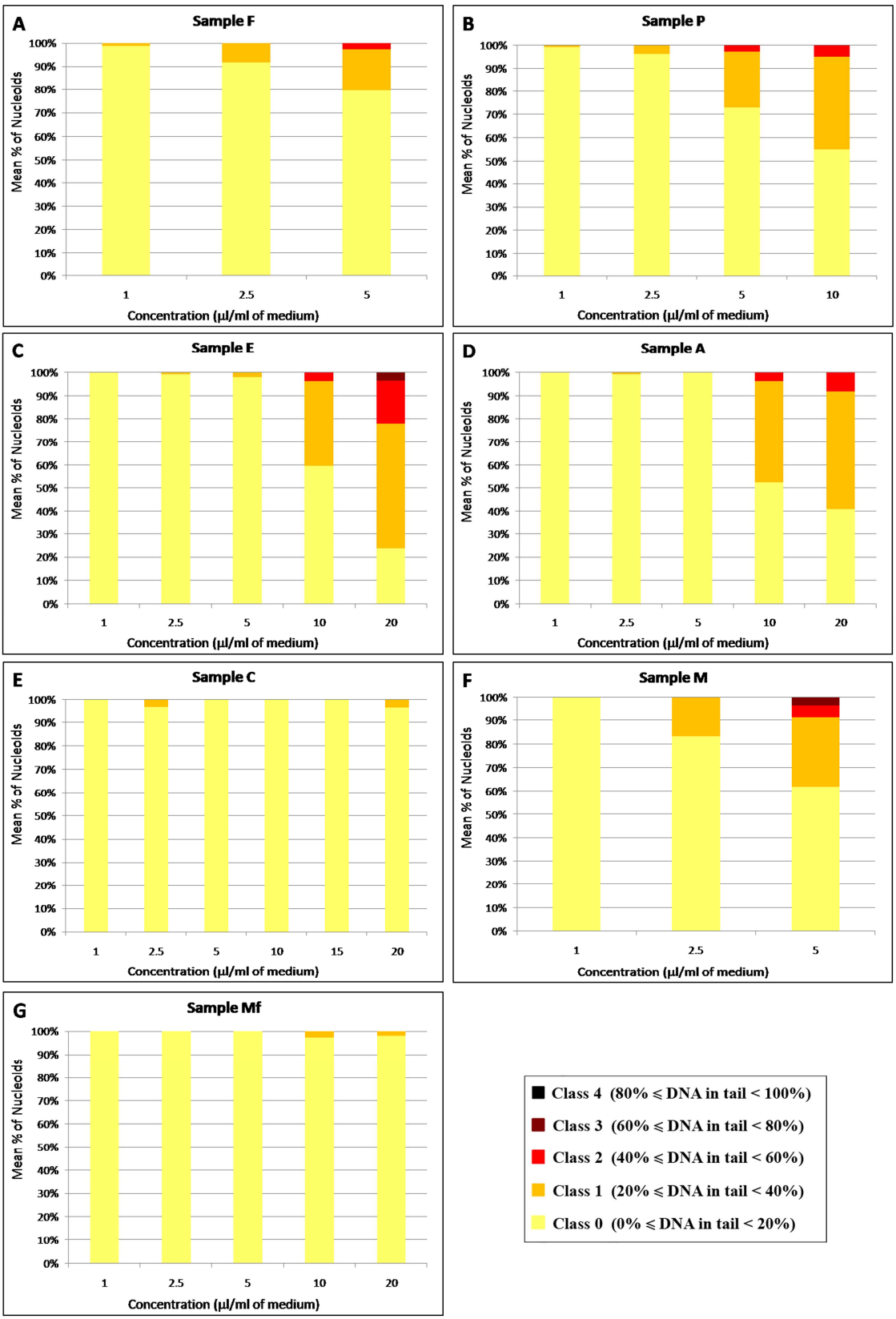

Figure 18 – Distribution of the mean percentage of observed nucleoids per classes. Classes were obtained from 3 independent experiments, according to the percentage of DNA in tail obtained for each extract sample, with FPG treatment. **A** – Sample F; **B** – Sample P; **C** – Sample E; **D** – Sample A; **E** – Sample C; **F** – Sample M; **G** – Sample Mf.

2.2. Micronucleus Assay

Following 24h exposure to samples F and M, a moderate increase in the frequency of micronuclei (MNi) can be observed in HepG2 binucleated cells, although without statistical significance (Figure 19). Sample C, failed to induce MNi.

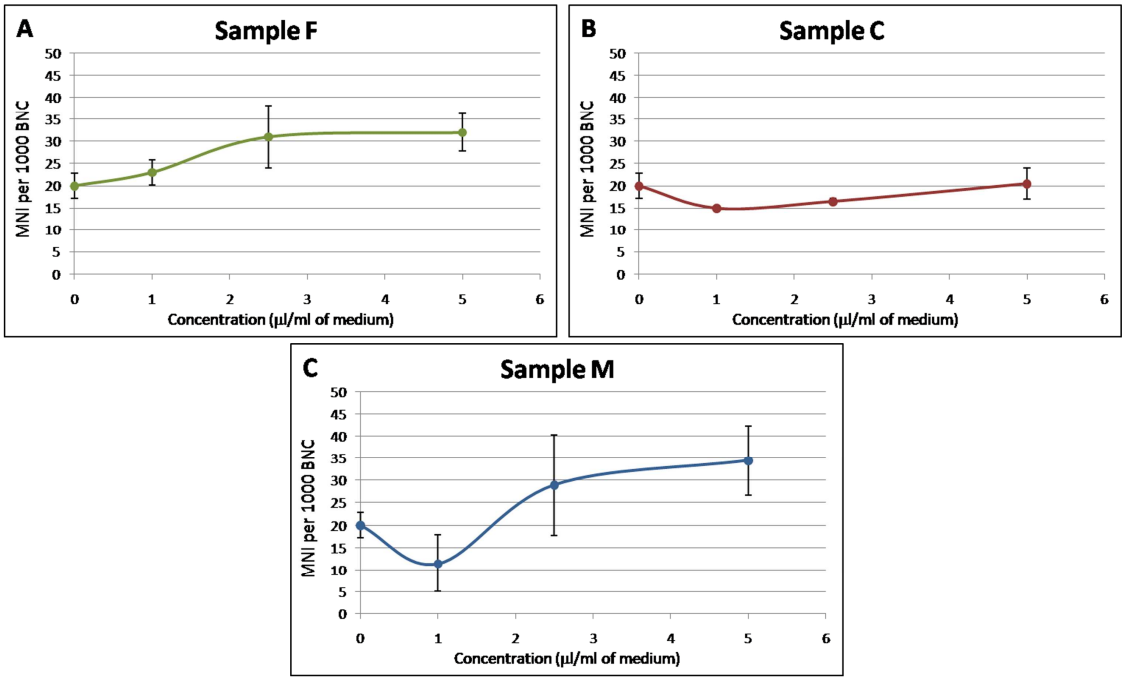

Figure 19 – Results for the micronucleus assay after 24h exposure to different concentrations of extract samples. Results are expressed as mean (± SD) of micronuclei per 1000 binucleated (BNC) cells. **A** – Sample F; **B** – Sample C; **C** – Sample M. Negative control DMSO at 0.5µl/ml.

When observing the frequency of NBUDs and NPBs, in HepG2 cells, compared with the solvent control, no significant induction could be observed for any sample (Table 15). Nevertheless, sample M shows an increase in NPB frequency at the highest concentration tested.

Table 15 – Total frequency of nuclear buds (NBUD) and nucleoplasmic bridges (NBPs) per 1000 binucleated cells observed in the micronucleus assay for extract samples F, C and M.

Sample	Concentration (µl/ml)	Total NBUDs	SD	Total NPBs	SD
DMSO	5.00	4	0.00	4	1.41
F	1.00	2	2.12	3	1.41
	2.50	2	2.83	5	3.54
	5.00	6	4.24	6	0.00
C	1.00	1	1.41	5	2.83
	2.50	4	1.41	5	2.83
	5.00	3	0.00	1	0.71
M	1.00	2	0.00	5	2.83
	2.50	5	0.71	4	1.41
	5.00	5	1.41	11	2.12

Results are expressed as mean (±SD) of two independent cultures.

All tested samples lead to a dose-related decrease in the CBPI and RI (Figures 20 and 21, respectively). RI values could be interpreted as an indication of cytotoxicity, and, much like the results obtained in the NR Assay, sample F is the most cytotoxic. Also, CBPI data reveals a decrease in cell cycle progression, more accentuated for sample F. The complete experimental data for these conditions is presented in Annex G.

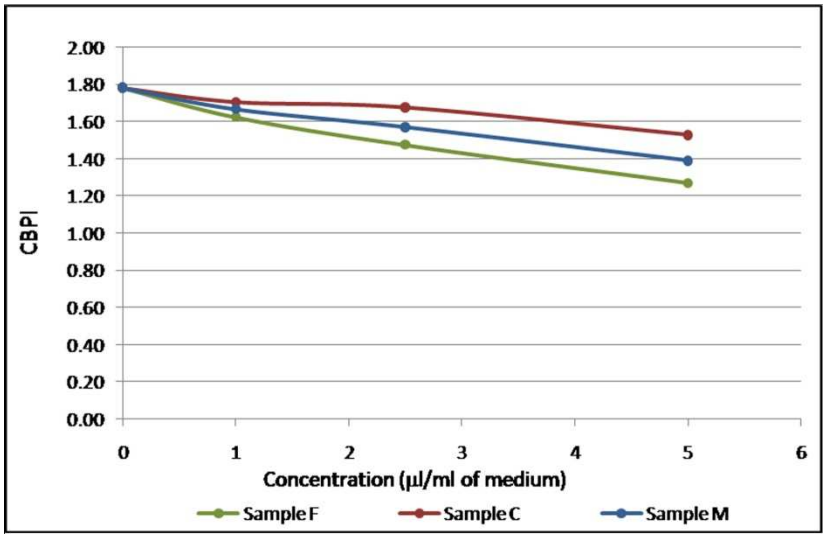

Figure 20 – Cytokinesis-block proliferation index (CBPI) calculated in the micronucleus assay for samples F, C and M.

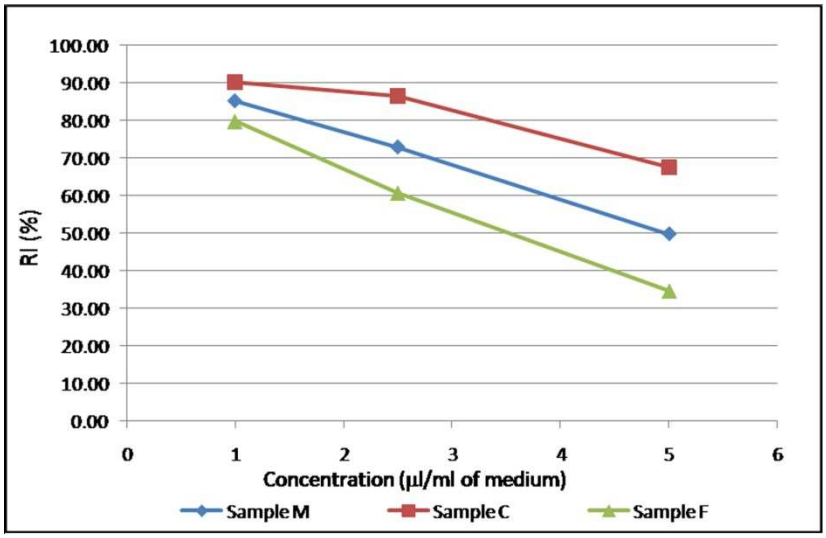

Figure 21 – Replication index (RI) calculated in the micronucleus assay for samples F, C and M.

DISCUSSION

As many estuaries in industrialized countries, the river Sado Estuary is affected by various sources of pollution, including heavy-industry and urban discharges, mining activities, agriculture and maritime traffic. It also remains a privileged site for fishing activities that are responsible for the supplying of consumable resources either locally or externally. Contaminants present in the estuary sediments can be accumulated in the edible parts of estuarine species or in local agricultural products and enter the human food chain, posing a public health problem, in addition to the ecological concern. Thus, the present study aimed at characterizing the cytotoxicity and genotoxicity, associated with the exposure to sediment contaminants, using a human liver-derived cell line.

Our first results of cytotoxicity assessment, following 2 and 4h exposure, revealed slight differences in cytotoxic effects between samples F (positive control, Sado Estuary), C (potentially clean sample, Sado Estuary) and M (sample from Mira Estuary). Although no significant cytotoxicity was observed in these experimental conditions, following 4h HepG2 cell exposure, sample F induced a more pronounced decrease in cell viability than the other two samples, which is in agreement with its previously characterized contaminant content (Annex A) [17]. These results led us to consider a longer exposure period, based on the knowledge that many of these contaminants, particularly PAHs, are promutagens, *i.e.* they need metabolic activation to be able to induce DNA damaging effects, and metabolites being more toxic than the original molecules [29, 32, 34, 35, 52].

After a longer exposure period (48h), all samples from the Sado Estuary were similarly ranked either based on their IC_{50} concentration or on the value of the minimum cell viability determined for the maximum concentration used (F > P > E > A, C). Considering their IC_{50}, as well as their dose-response curves, samples F and P (Figures 13A and 13B) appear to have a higher contaminant-driven cytotoxic potential than sample E (Figure 13C), whose IC_{50} corresponded to a 2- to 4-fold higher concentration. However, the difference in the estimated IC_{50}, suggests that sample F could contain a higher level of cytotoxic contaminants than sample P (IC_{50}=5.09 and 9.24µl/ml, for F and P, respectively). Nevertheless, since samples F and P were both collected in nearby sites, we hypothesize that sample P might have similar contaminants at different levels. As for samples A and C (Figures 13D and 13E), the reduction of cell viability observed, at the highest tested concentration, was probably due to some

cytotoxicity of the solvent (DMSO), or very low levels of contaminants that are toxic to human cells.

In summary, following 48h exposure to the different sediment extracts, the results from cytotoxicity analysis suggests different possible levels of overall contamination, in the Sado Estuary: sites of possible low contamination (sites A and C), a site of heavy contamination (site P, which is comparable to the previously studied site F) and a site of moderate contamination (site E). The combinations of three types of factors, namely, sources of pollutants, sediment geochemistry and hydrodynamics will most likely determine the differential cytotoxicity of sediments, according to the collection site. In fact, samples that induce similar cytotoxic dose-responses, in HepG2 cells, have similar associated anthropogenic pressures. Our results of cytotoxicity are in agreement with previous studies by Costa *et al.* (2010), that also revealed mortality in *S. senegalensis* exposed to sediment F [17]. Moreover, the difference in the cytotoxic potency, between samples from the northern margin, might also be explained by differences in hydrodynamics of the three sites, which would be reflected in the amount or nature of contaminants retained in the sediments (Table 7). Site P seems to suffer a higher influence of river currents than site F, a site with a very low hydrodynamic, therefore more prone to accumulate by deposition particles to which both hydrophilic (metals) and hydrophobic (PAHs) contaminants may be adsorbed to. In contrast, sample C, collected further away from the contaminant hotspots, but still near the northern urban and industrial area of Setúbal revealed no significant contaminant-driven cytotoxicity. The intersection of different factors in sample C's collection area such as sediment characteristics (mostly sand), high hydrodynamic pressures, from both river and ocean, and low fine fraction deposition might account for the presence of low levels of contamination. This points to the possibility of using this site as a potential reference for the Sado Estuary in further studies, but also suggests that contaminants discharged into the northern area of the estuary might be predominantly retained in sediments along the estuarine margin.

As for the southern margin, samples E and A exhibited lower cytotoxic effects in HepG2 cells. Similar dose-response curves were obtained for these two samples, which would indicate a lower level, or different type of contamination surrounding the southern area of the Sado Estuary. As far as it is known, this area can be influenced by chemicals used in either agriculture practices by the local population (site E) or intensive rice cultures (site A). Considering the inexistence of heavy industry in the

southern margin, significant PAH contamination should not be expected in this area, and metal contamination should be mainly due to natural pressures, but both groups of contaminants could be transported along the Alcácer Channel into the estuary. Nevertheless, through agricultural practices, pesticides and other agricultural products might be drained into the estuary, and retained in its sandy-muddy sediments, which in part may be responsible for the cytotoxicity observed.

As for the Mira Estuary, the two collected samples also revealed different cytotoxic potency, either low (site Mf, Figure 14B) or high (site M, Figure 14A). The cytotoxicity of sample M is comparable to that obtained for samples F and P. However, taking into account the results obtained following a 4h exposure period, we suggest that there could be a difference in sediment contamination type, because the extract from sediment sample F was cytotoxic, while that from sample M was not. This could be explained by sample M sampling location, which is near an aquaculture facility (a potential direct source of pollutants released in this estuary), along with the low hydrodynamics of the area, thus facilitating deposition of fine particles and organic matter, that acts as a trap for discharged contaminants. Also, the maximum contamination values observed in the Mira Estuary presented in Tables 4 and 5 were obtained near sample M's collection site, suggesting that the contaminants found in the sediments from this site might also contribute to its cytotoxicity. In fact, total SQG-Q values for the maximum values of the Mira Estuary are very close to a moderate risk association (Table 6). Reference sample Mf revealed no significant cytotoxic induction and its cytotoxic pattern is very similar to the solvent control. Also, the minimum contamination previously observed in the Mira Estuary revealed low levels of organic and inorganic contaminants (minimum values were obtained near sample Mf sampling area; Table 4 and 5), and a low risk associated with the estuary's sediment quality near this area (Table 6).

Through the standard alkaline comet assay performed for samples F, C and M, no significant genotoxicity could be observed following 48h exposure (Figure15). This led us to increase the comet assay sensitivity for the genotoxicity assessment of these samples, probably containing a complex mixture of chemicals, and to account for the potential effect of oxidative stress on DNA damage, by including FPG treatment, which allows the conversion of oxidized purines in DNA breaks [37, 56].

Much like the cytotoxicity evaluation, results obtained for DNA damage through the comet assay revealed different patterns of genotoxicity, for the several samples

under analysis. When observing the overall DNA damage obtained (accounting or not for oxidative DNA damage) samples exhibit the same similarities as in the NR assay.

Our results show that, at the highest concentration tested, extracts from Sado samples F, P and E, induced a significant level of DNA damage (in the absence of FPG treatment), in HepG2 cells, comparatively to the solvent control (Figures 16A, 16B, 16C, respectively). Noteworthy, these samples were also those that showed a lower IC_{50} value, when tested for their cytotoxicity, *i.e.* they are the most genotoxic and cytotoxic. This finding reinforces the previous suggestion that these samples might exhibit the highest levels of contamination, or the most genotoxic contaminants. In contrast, samples A and C did not reveal significant genotoxic damage, suggesting a low level of contamination, which is also consistent with the cytotoxicity data (Figures 16D and 16F, respectively). As expected, in the Mira Estuary, sample Mf gave negative results (Figure 17B), while sample M also induced a significant level of DNA damage (Figure 17A).

Samples P and F, from the Sado Estuary, appear to have similar genotoxic patterns but at different concentrations. Both samples were able to induce significant DNA damage at concentrations near their IC_{50}, when compared with the respective solvent controls. Nevertheless, at IC_{50} concentration, sample P induced higher levels of DNA damage and, especially, of oxidative DNA damage, than sample F. This may suggest a different type of contaminants present in the two samples, or it might reflect, for sample F, the inactivation of some contaminants due to long term preservation (sample collected in 2007). So, even though sample F is slightly more cytotoxic, sample P might contain more or different contaminants able to induce DNA damage. Other studies on marine sediments have also shown that doses that induce similar cytotoxicity do not necessarily induce similar genotoxicity [11]. This might be explained by the complexity of the mixture and the contaminants' ability to interact with each other. When observing contaminant content of sample F (Annex A), several contaminants that are known to induce direct DNA damage (*e.g.*, PAHs), and indirect DNA damage (*e.g.*, metals), are present at concentrations above the threshold effect level guideline. Taking into account our results, we hypothesize that sample P might contain higher levels, or different proportions, of these particular contaminants. In fact, higher levels of these particular organic and inorganic contaminants have been observed in sediments collected in different sites of the Sado Estuary (Tables 4 and 5) [2]. Other studies on marine sediments have also associated the presence of high content of genotoxic PAHs

or metals to the surrounding industrial, harbor associated, and urban areas [8, 11, 65], and dose-related genotoxicity has been reported using sediment extracts from these types of areas [12]. Sample C did not induce significant DNA damage in the experimental conditions used, at any concentration, reinforcing the apparent absence of contaminants with a cytotoxic or genotoxic effect in human cells. The overall genotoxicity results for samples A and E revealed similar patterns, and the combined results from the NR and comet assays show that the type of contamination might be similar but at different levels, given that, at comparable concentrations, sample E exhibited higher cytotoxic and genotoxic potential than sample A. Considering that sample A was collected near an agricultural discharge channel, and very near the intensive rice field cultures, it would be expected that its extract was more cytotoxic and genotoxic than that from sample E. The later was collected further downstream from sample A's collection site, which could possibly indicate that contaminants are transported by the currents from the southern channel (Alcácer Channel), and allowed to settle near the fishing grounds where hydrodynamics are lower (site E) [2]. The difference observed between samples may also be the result of the contaminant extraction that, although it is considered as the broader extraction method [1], might not be able to extract some of the chemicals used in intensive agricultural practices. Also, we suggest that sample A might contain other types of contaminants presenting low cytotoxic and genotoxic potential, such as pesticides. In fact, the genotoxic potential for agrochemical ingredients is generally low, and they have been shown to give positive results in few genotoxicity tests [66].

Moreover, with the exception of sample C, all samples revealed significant additional oxidative DNA damage (revealed as FPG sensitive sites), when compared with the solvent control. This observation might suggest that these complex mixtures of contaminants may have a common mechanism of inducing DNA damage through oxidative stress, even though the level of oxidative DNA damage is different between samples. As previously mentioned, metals, PAHs, PCBs and DDTs have been associated with the formation of ROS, or metabolites with oxidative potential [24, 30, 32, 34]. Our data suggest that the presence of metal and PAH contamination hotspots in the Sado Estuary's northern margin might account for much of the oxidative DNA damage observed, in samples P and F, as it seems to be a commonly proposed mechanism of DNA damage induction by these agents [24]. Costa *et al* (2010) observed a relationship between blood plasma lipid peroxidation and genotoxicity, in erythrocytes

of fish exposed to sediment F, suggesting that oxidative stress might mediate the genotoxic effect of that sediment [17]. Noteworthy, in the present study, samples E and A, exhibited the highest potency to induce oxidative DNA damage, which might be associated with the presence of pesticides [67]. Although the use of DDTs has been banned for decades, sizable amounts of persistent DDTs have been observed in sample F (Annex A), which is located on the opposite side of the estuary [17], which suggests that these sediments contain traceable amounts of pesticides transported from upstream grounds. Thus, it is plausible that the area surrounding samples E and A collection sites, is contaminated with pesticides, and/or other chemicals used in agriculture. Also, pesticides discharged into the Alcácer Channel might have been transported onto the estuary, and persistently retained in the sediments from the southern side of the estuary. The difference in cytotoxicity and genotoxicity observed between both samples could be due to the presence of a higher content of genotoxic pesticides in sample E, such as, for example, organophosphorous pesticides, that have been shown to induce DNA damage, in HepG2 cells, through the alkaline comet assay, and reduce cell proliferation [68], or to the different retention capacity of the sediments in the two locations.

In the Mira Estuary, sample Mf was unable to induce significant genotoxicity and only produced low levels of oxidative DNA damage at the highest tested concentration. This reinforces its characterization as the reference site used in this study, and for further studies. In general, the results for sample Mf appear to be in accordance with the low anthropogenic pressures from the small nearby urban area of Vila Nova de Milfontes, and low environmental pressures. Sample M was able to produce significant genotoxicity (with or without FGP treatment), which suggests again that this site might be moderately contaminated. Even though agricultural activities are lower than in the Sado Estuary, the fact that higher values of *pp'*DDD and *pp'*DDE were measured in the Mira Estuary, and that DDTs are still quantifiable (Table 5), also leads us to consider that these persistent pesticides, or other agricultural products, might contribute for the cytotoxicity and genotoxicity observed in sample M.

As for the micronucleus assay data, samples F and M were able to induce a moderate increase in the MNi frequency (Figures 19A and 19C, respectively), at the highest tested concentration, while sample C did not induce MNi (Figure 19B). This further reinforces our hypothesis that sample M is probably contaminated. When observing the CBPI and RI results for both samples (Figures 20 and 21, respectively), F

appears to have a higher cytotoxic induction than sample M. These preliminary results were consistent with the cytotoxicity results obtained in the NR assay.

Our results of the cytotoxic and genotoxic potential of sediments from the Sado Estuary suggest that the overall contamination of the estuary appears to be differential according to the estuarine areas. Previous results have already shown this spatial difference in the estuary contamination, which is mainly explained by the anthropogenic pressure contribution in each area [2, 45], and differences in the levels of contaminants reflected in the maximum and minimum concentrations of contaminants [42]. The industrial, urban and mining activities on the northern margin would account for most of the area's contamination, consisting of PAHs, PCBs and metals that are discharged in the estuary [17, 39, 41]. Our results from samples P, and F (previously collected for other studies) corroborate this observation, either by their high cytotoxic induction or by their ability to induce genotoxic damage, that might be attributed to a moderate level of PAH contamination. In fact, previous reports on other estuaries have attributed direct genotoxic damage to PAH mixtures present in estuarine sediments [69]. Also, a correlation between PAH concentrations and sediment toxicities has already been observed when testing marine sediments, with higher PAH content corresponding to higher toxicity [11]. Nevertheless, a report on marine sediments, from Croatia, showed that a sediment with the highest total PAH content might not be responsible for the highest induction of DNA damage [65], which may reflect the previously mentioned interactions in such a complex mixture. Despite this information, hazardous amounts of known genotoxic PAH have been reported in the Sado Estuary [17, 41], and previous studies on aquatic species, exposed to sediments from the northern margin of the estuary, demonstrated distinct patterns of histopathological conditions, associated with the anthropogenic activities in this area [47, 48]. Although PAHs might be high contributors to toxicity, they should not be considered the unique source of genotoxicity. As they are produced through the combustion of organic material, they are not expected to be found in high quantities in the southern margin of the estuary, where the main activities consist of agriculture and fishing. Nevertheless, results from samples E and A revealed predominantly high induction of oxidative DNA damage. We propose that this genotoxic effect could be associated with either natural metals, or products used and discharged by local agricultural activities, given that these contaminants may commonly produce ROS. The presence of persistent pesticides used in agricultural

practices could account for part of the high levels of oxidative DNA damage observed for samples in this area [32], but contribution of other products of agricultural practice or even high metal contamination associated with old mining activities and outcrop erosion in the Alcácer Channel [2], should not be excluded.

Different assays generated complementary information for the characterization of the sediments' cytotoxicity and genotoxicity. The extracts from the Sado Estuary sediment samples that exhibited a low cytotoxic effect (samples A and E) were those that were able to induce the highest levels of oxidative DNA damage. This means that for sediment contaminants a direct relationship between cytotoxic and genotoxic potencies might not be observed. Thus, in order to evaluate the risk of exposure to sediment contaminants, to human health, multiple assays should be considered, as information from only one does not fully reveal the toxic potential of sediment samples [1, 13]. Noteworthy, all samples tested in this study were collected in frequently used fishing sites, by the local fishing community, particularly from the southern margin of the Sado Estuary. Moreover, the aquatic species are consumed by the population and are a major source of food income in this area. Additionally, this catch might be distributed locally and nationally, increasing the potential exposure to contaminants from the Sado Estuary. It is also important to state that these contaminants are present in the Sado Estuary waters, which are also used for agricultural activities, providing another source of human exposure, through direct contact and consumption of agricultural products watered with potentially contaminated waters. Our results show that the risk associated with the exposure to sediments from the Sado Estuary should not be ignored. Exposure to sediments from the northern industrialized and urban margin of estuary resulted in direct induction of genotoxic damage, but exposure to sediments from the southern agricultural margin resulted, predominantly, in indirect genotoxic damage. Other reports on estuarine contamination have associated the mutagenic potential of estuarine sediments with heavy industrialized area discharges, for example, in the United Kingdom [18], in France [13], or Korea [16]. Previous studies on aquatic species have shown their ability to bioaccumulate the present contaminants, after exposure to sediments from the northern margin of the Sado Estuary [46]. If these contaminants are available, and capable of being bioaccumulated in edible parts of local aquatic species, human exposure through the food chain might not be negligible. This hypothesis comes in light of the fact that most of the contaminants, present in the Sado Estuary sediments, have been associated to the occurrence of cancer and other diseases. This is of particular

concern taking into the account that these contaminants can be transported and distributed along the Sado Estuary, accumulated in the estuary sediments, and through incorporation and adsorption, can be "stored" and repeatedly released, contributing to the availability to local aquatic species. Thus, there is an inherent environmental, ecological and human health, risk associated with the Sado Estuary sediment contamination that must be properly assessed.

Even though it was beyond the scope of this study, the assays performed here are insufficient for establishing an association between a single, or group, of contaminants and the observed cytotoxicity and genotoxicity, given the fact that sediment chemical analysis and sediment extraction by a fractionated method were not performed yet. Nevertheless, the overall observed effects cannot be ignored, and coupled with chemical analysis of sediment samples, should contribute for assessing the risk associated with the direct or indirect exposure to contaminated estuarine sediments [1]. In this study, the biomarkers analyzed demonstrated to be useful in assessing the potential risk of contaminated estuarine sediments. Furthermore, chemical analysis might shed light on a possible predominant group of contaminants present in the tested samples, that might be associated to the observed effects but, as previously mentioned, interactions between contaminants, in such a complex mixture, should not be excluded. Also, a fractionated contaminant extraction from sediment samples, either by groups of contaminants or by contaminant polarity, might allow the establishment of an association between a given class of contaminants and the observed cytotoxicity and genotoxicity, in human cells.

FUTURE PERSPECTIVES

Our study has revealed significant cytotoxic and genotoxic effects, in a human-derived liver cell line, driven by contaminants present in the Sado Estuary sediments. This study constitutes part of a broader project, and further work is needed in order to understand and assess the risk associated with the exposure to contaminated sediments from this estuary. A full genotoxic profile should be characterized on the collected sediment samples through, for example, the micronucleus assay, which could not be performed in this work due to time limitations. A chemical analyses and characterization, in terms of concentrations and types of contaminants present in these samples, is needed to try establishing an association between the cytotoxic and genotoxic effects observed and a particular or predominant group of contaminants. Also, as proposed by Chen and White (2004) [1], sediment sample extract preparation should be attempted by a fractionated method, with at least two separate extractions with solvents of different hydrophobicities, and cytotoxic and genotoxic assays should be performed, on the different fractions, in order to complement, and hopefully better understand, the results observed here following a broad range contaminant extraction method. We proposed that the formation of ROS could be a common mechanism behind the induction of DNA damage by part of the sediment contaminants, and attempted a study on oxidative DNA damage with the use of FPG in the alkaline comet assay. However, other endonucleases can be used, such as endonuclease III to detect oxidized pyrimidines, and possibly reveal a different genotoxic profile [57]. As previously mentioned, contaminated sediment exposure can be linked to a potential risk for human health, through the consumption of local aquatic species, or agricultural products, therefore a study on local aquatic species, and a study on a local human population, from the Carrasqueira village, in the southern margin of the Sado Estuary, are underway. It is expected that the information obtained with the integration of these approaches will provide a better understanding of the toxicological potential of contaminated sediments from the Sado Estuary, as well as contribute to assess the associated environmental and human health risks.

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ANNEXES

ANNEX A - Physico-chemical characterization and contamination profiles of the sediment sample F. The TEL and PEL sediment quality guidelines were obtained from Macdonald *et al.* (1996) [43]. (Adapted from [17])

			Site	F		
			TOM (FF)	10.2		
			FF (%)	95.6		
			Eh (mV)	−300	SQGs	
Contaminant					*TEL*	*PEL*
Metallic/other (μg g^{-1} sediment dw)		Non-metal	Se	1.21 ± 0.02	*NG*	*NG*
		Metalloid	As	23.98 ± 0.48*	7.24	41.6
		Metal	Cd	0.26 ± 0.01	0.68	4.21
			Co	13.94 ± 0.28	*NG*	*NG*
			Cr	80.73 ± 1.61*	52.3	160
			Cu	172.72 ± 3.45**	18.7	108
			Hg	0.69 ± 0.01*	0.13	0.7
			Mn	464.34 ± 9.29	*NG*	*NG*
			Ni	33.30 ± 0.67*	15.9	42.8
			Pb	55.19 ± 1.10*	30.2	112
			Zn	364.83 ± 7.30**	124	271
			SQG-Q Metallic	***0.79***		
Organic (ng g^{-1} sediment dw)	PAH	3-ring	acenaphthylene	2.38 ± 0.40	5.87	128
			acenaphthene	12.25 ± 2.08*	6.71	88.9
			fluorene	15.33 ± 2.61	21.2	144
			phenanthrene	63.87 ± 10.86	86.7	544
			anthracene	21.00 ± 3.57	46.9	245
		4-ring	fluoranthene	315.71 ± 53.67*	113	1494
			pyrene	263.18 ± 44.74*	153	1398
			benzo(a)anthracene	81.25 ± 13.81*	74.8	693
			chrysene	41.06 ± 6.98	108	846
		5-ring	benzo(b)fluoranthene	98.00 ± 16.66	*NG*	*NG*
			benzo(k)fluoranthene	30.76 ± 5.23	*NG*	*NG*
			benzo(e)pyrene	74.95 ± 12.74	*NG*	*NG*
			benzo(a)pyrene	101.86 ± 17.32*	88.8	763
			perylene	96.29 ± 16.37	*NG*	*NG*
			dibenzo(a.h)anthracene	13.32 ± 2.26*	6.22	135
		6-ring	indeno(1.2.3-cd)pyrene	82.06 ± 13.95	*NG*	*NG*
			benzo(g.h.I)perylene	51.93 ± 8.83	*NG*	*NG*
			tPAH	**1 365.20 ± 232.08**	**1684**	**16770**
			SQG-Q PAHs	***0.11***		
	PCB	Trichlorinated	18	0.27 ± 0.05	*NG*	*NG*
			26	1.80 ± 0.31	*NG*	*NG*
			31	0.26 ± 0.04	*NG*	*NG*
		Tetrachlorinated	44	0.17 ± 0.03	*NG*	*NG*
			49	0.13 ± 0.02	*NG*	*NG*
			52	0.10 ± 0.02	*NG*	*NG*
		Pentachlorinated	101	0.25 ± 0.04	*NG*	*NG*
			105	0.26 ± 0.04	*NG*	*NG*
			118	0.55 ± 0.09	*NG*	*NG*
		Hexachlorinated	128	0.26 ± 0.04	*NG*	*NG*
			138	0.71 ± 0.12	*NG*	*NG*
			149	0.05 ± 0.01	*NG*	*NG*
			151	0.77 ± 0.13	*NG*	*NG*
			153	0.98 ± 0.17	*NG*	*NG*
		Heptachlorinated	170	0.20 ± 0.03	*NG*	*NG*
			180	0.73 ± 0.12	*NG*	*NG*
			187	0.29 ± 0.05	*NG*	*NG*
			194	0.12 ± 0.02	*NG*	*NG*
			tPCB	**7.91 ± 1.34**	**21.6**	**189**
			SQG-Q PCBs	***0.04***		
	DDT		*pp'*DDD	0.37 ± 0.06	1.22	7.81
			*pp'*DDE	< d.l.	2.07	374
			*pp'*DDT	< d.l.	1.19	4.77
			tDDT	0.37 ± 0.06	**3.89**	**51.7**
			SQG-Q DDTs	***0.02***		
			SQG-Q Organic	***0.09***		
			SQG-Q Total	***0.33***		
			Overall rating	**Moderately impacted**		

< d.l., below detection limit; *, value above TEL; **, value above PEL; Eh, sediment redox potential; FF, sediment fine fraction (particle size < 0.63 μm); NG, no guideline available; PEL, probable effects level guideline; SQG-Q, Sediment Quality Guideline quotient; TEL; threshold effects level guideline; TOM, total organic matter. Contaminant concentration ranges indicate the standard quantification error.

ANNEX B – Experimental data obtained in the neutral red assay following a 2h exposure period to sediment extracts.

Table B1 – Neutral red assay data, of 3 independent experiments, for sample F after 2h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
F	0.00	0.358	100.00	0.336	100.00	0.348	100.00	0.347	0.01	100.00	0.00
	0.10	0.441	123.28	0.347	103.27	0.325	93.48	0.371	0.04	106.68	8.77
	0.60	0.351	98.14	0.316	94.05	0.359	103.16	0.342	0.01	98.45	2.64
	1.25	0.336	93.95	0.308	91.77	0.348	100.00	0.331	0.01	95.24	2.46
	2.50	0.396	110.61	0.307	91.27	0.306	88.02	0.336	0.03	96.63	7.05
	5.00	0.398	111.17	0.333	99.21	0.256	73.73	0.329	0.04	94.70	11.04
	10.00	0.372	103.91	0.306	90.97	0.250	71.91	0.309	0.04	88.93	9.29
	15.00	0.337	94.13	0.270	80.26	0.252	72.58	0.286	0.03	82.32	6.31
	20.00	0.247	68.99	0.254	75.60	0.185	53.31	0.229	0.02	65.97	6.61

Table B2 – Neutral red assay data, of 3 independent experiments, for sample C after 2h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
C	0.00	0.358	100.00	0.336	100.00	0.348	100.00	0.347	0.01	100.00	0.00
	0.10	0.410	114.62	0.297	88.39	0.329	94.53	0.345	0.03	99.18	7.92
	0.60	0.386	107.82	0.333	99.01	0.401	115.44	0.373	0.02	107.42	4.75
	1.25	0.362	101.12	0.324	96.53	0.457	131.35	0.381	0.04	109.67	10.92
	2.50	0.402	112.29	0.348	103.67	0.385	110.83	0.379	0.02	108.93	2.66
	5.00	0.449	125.42	0.328	97.62	0.337	96.84	0.371	0.04	106.62	9.40
	10.00	0.391	109.31	0.324	96.43	0.395	113.61	0.370	0.02	106.45	5.16
	15.00	0.356	99.53	0.352	104.86	0.401	115.44	0.370	0.02	106.61	4.67
	20.00	0.410	114.53	0.355	105.65	0.358	103.07	0.374	0.02	107.75	3.47

Table B3 – Neutral red assay data, of 3 independent experiments, for sample M after 2h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
M	0.00	0.358	100.00	0.336	100.00	0.348	100.00	0.347	0.01	100.00	0.00
	0.10	0.411	114.71	0.481	143.15	0.355	102.21	0.416	0.04	120.02	12.12
	0.60	0.398	111.08	0.316	94.15	0.485	139.60	0.400	0.05	114.94	13.26
	1.25	0.354	98.98	0.292	87.00	0.405	116.59	0.351	0.03	100.86	8.59
	2.50	0.358	99.91	0.346	102.88	0.371	106.81	0.358	0.01	103.20	2.00
	5.00	0.425	118.81	0.349	103.77	0.423	121.67	0.399	0.03	114.75	5.55
	10.00	0.393	109.78	0.273	81.35	0.350	100.77	0.339	0.04	97.30	8.39
	15.00	0.286	79.80	0.228	67.86	0.274	78.91	0.263	0.02	75.52	3.84
	20.00	0.354	98.88	0.251	74.60	0.233	67.11	0.279	0.04	80.20	9.59

Table B4 – Neutral red assay data, of 3 independent experiments, for DMSO after 2h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
DMSO	0.00	0.358	100.00	0.336	100.00	0.348	100.00	0.347	0.01	100.00	0.00
	0.10	0.371	103.54	0.324	96.53	0.423	121.76	0.373	1.01	107.28	7.52
	0.50	0.356	99.44	0.394	117.16	0.332	95.40	0.360	2.01	104.00	6.68
	1.00	0.385	107.64	0.316	93.95	0.314	90.41	0.338	3.01	97.33	5.25
	5.00	0.397	110.89	0.327	97.32	0.406	116.87	0.377	4.01	108.36	5.78
	10.00	0.411	114.80	0.305	90.87	0.412	118.50	0.376	5.01	108.06	8.66
	20.00	0.372	103.91	0.351	104.37	0.390	112.27	0.371	6.01	106.85	2.71

ANNEX C – Experimental data obtained in the neutral red assay following a 4h exposure period to sediment extracts.

Table C1 – Neutral red assay data, of 3 independent experiments, for sample F after 4h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
F	0.00	0.387	100.00	0.287	100.00	0.279	100.00	0.318	0.03	100.00	0.00
	0.10	0.317	81.93	0.295	103.02	0.339	121.51	0.317	0.01	102.15	11.43
	0.60	0.382	98.71	0.214	74.53	0.239	85.54	0.278	0.05	86.26	6.99
	1.25	0.385	99.40	0.252	88.02	0.273	97.85	0.303	0.04	95.09	3.56
	2.50	0.351	90.62	0.223	77.67	0.227	81.36	0.267	0.04	83.22	3.85
	5.00	0.307	79.35	0.222	77.44	0.279	100.00	0.269	0.03	85.60	7.22
	10.00	0.292	75.47	0.158	55.23	0.177	63.32	0.209	0.04	64.68	5.88
	15.00	0.211	54.39	0.140	48.84	0.155	55.44	0.168	0.02	52.89	2.05
	20.00	0.180	46.56	0.157	54.65	0.127	45.52	0.155	0.02	48.91	2.89

Table C2 – Neutral red assay data, of 3 independent experiments, for sample C after 4h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
C	0.00	0.387	100.00	0.287	100.00	0.279	100.00	0.318	0.03	100.00	0.00
	0.10	0.369	95.35	0.332	115.81	0.254	91.16	0.319	0.03	100.78	7.62
	0.60	0.370	95.61	0.268	93.60	0.305	109.32	0.315	0.03	99.51	4.94
	1.25	0.382	98.54	0.236	82.33	0.347	124.37	0.322	0.04	101.75	12.24
	2.50	0.364	93.98	0.254	88.72	0.306	109.56	0.308	0.03	97.42	6.26
	5.00	0.344	88.73	0.309	107.91	0.323	115.65	0.325	0.01	104.09	8.00
	10.00	0.400	103.27	0.307	107.09	0.392	140.50	0.366	0.03	116.96	11.82
	15.00	0.376	96.99	0.295	102.79	0.385	138.11	0.352	0.03	112.63	12.85
	20.00	0.386	99.57	0.337	117.44	0.342	122.70	0.355	0.02	113.24	7.00

Table C3 – Neutral red assay data, of 3 independent experiments, for sample M after 4h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
M	0.00	0.387	100.00	0.287	100.00	0.279	100.00	0.318	0.03	100.00	0.00
	0.10	0.362	93.55	0.354	123.49	0.329	117.92	0.348	0.01	111.65	9.19
	0.60	0.349	90.02	0.284	99.19	0.368	131.90	0.334	0.03	107.03	12.71
	1.25	0.338	87.18	0.310	108.02	0.340	121.74	0.329	0.01	105.65	10.05
	2.50	0.319	82.44	0.284	99.07	0.305	109.32	0.303	0.01	96.94	7.83
	5.00	0.313	80.81	0.371	129.30	0.376	134.89	0.353	0.02	115.00	17.17
	10.00	0.324	83.56	0.287	100.23	0.326	116.73	0.312	0.01	100.17	9.57
	15.00	0.270	69.79	0.218	76.16	0.300	107.41	0.263	0.02	84.45	11.62
	20.00	0.249	64.20	0.213	74.30	0.228	81.72	0.230	0.01	73.41	5.08

Table C4 – Neutral red assay data, of 3 independent experiments, for DMSO after 4h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
DMSO	0.00	0.387	100.00	0.287	100.00	0.279	100.00	0.318	0.03	100.00	0.00
	0.10	0.392	101.29	0.354	123.37	0.380	136.08	0.375	0.01	120.25	10.16
	0.50	0.406	104.73	0.331	115.35	0.331	118.52	0.356	0.03	112.87	4.17
	1.00	0.357	92.25	0.338	117.79	0.379	135.84	0.358	0.01	115.30	12.64
	5.00	0.371	95.78	0.305	106.28	0.279	100.12	0.318	0.03	100.73	3.05
	10.00	0.362	93.46	0.327	113.95	0.377	135.13	0.355	0.01	114.18	12.03
	20.00	0.365	94.15	0.278	96.86	0.312	111.83	0.318	0.03	100.95	5.50

ANNEX D – Experimental data obtained in the neutral red assay following a 48h exposure period to sediment extracts.

Table D1 – Neutral red assay data, of 5 independent experiments, for sample F after 48h exposure.

	Concentration	1st Assay		2nd Assay		3rd Assay		4th Assay		5th Assay		Mean		Mean	
Sample	(µl/ml)	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	SE	$\%_{Viability}$	SE
F	0.00	0.428	100.00	0.573	100.00	0.576	100.00	0.559	100.00	0.690	100.00	0.565	0.05	100.00	0.00
	0.10	-	-	0.412	71.84	0.636	110.36	0.576	102.98	-	-	0.541	0.07	95.06	11.80
	0.20	-	-	0.466	81.33	0.640	111.11	0.606	108.35	-	-	0.571	0.05	100.26	9.50
	0.40	-	-	0.423	73.82	0.580	100.69	0.576	103.04	-	-	0.526	0.05	92.52	9.37
	0.60	-	-	0.382	66.72	0.553	96.01	0.533	95.29	-	-	0.489	0.05	86.01	9.64
	0.80	-	-	0.501	87.49	0.534	92.65	0.426	76.27	-	-	0.487	0.03	85.47	4.84
	1.00	-	-	0.427	74.52	0.513	89.12	0.541	96.84	-	-	0.494	0.03	86.83	6.54
	1.25	0.324	75.72	0.301	52.53	-	-	0.498	89.09	-	-	0.374	0.06	72.45	10.68
	1.50	-	-	-	-	0.427	74.07	0.436	78.06	0.539	78.20	0.467	0.04	76.78	1.35
	2.00	-	-	-	-	0.426	73.90	0.322	57.54	0.429	62.25	0.392	0.04	64.57	4.86
	2.50	0.258	60.23	0.336	58.64	0.447	77.66	-	-	-	-	0.347	0.05	65.51	6.09
	3.00	-	-	-	-	0.347	60.24	0.518	92.73	0.433	62.78	0.433	0.05	71.92	10.43
	3.50	-	-	-	-	0.361	62.67	0.391	69.89	0.424	61.53	0.392	0.02	64.70	2.62
	3.75	0.203	47.32	0.311	54.28	-	-	0.342	61.18	-	-	0.285	0.04	54.26	4.00
	4.00	-	-	-	-	0.307	53.30	0.309	55.34	0.307	44.51	0.308	0.00	51.05	3.32
	4.50	-	-	-	-	0.255	44.21	0.429	76.74	0.391	56.65	0.358	0.05	59.20	9.48
	5.00	0.175	40.86	0.226	39.44	0.256	44.39	-	-	-	-	0.219	0.02	41.56	1.47
	6.25	0.133	30.97	0.233	40.66	-	-	0.360	64.34	-	-	0.242	0.07	45.33	9.91
	7.50	0.097	22.72	0.120	20.88	-	-	0.291	52.00	-	-	0.169	0.06	31.87	10.08
	8.75	0.107	25.06	0.151	26.29	-	-	0.226	40.37	-	-	0.161	0.03	30.57	4.91
	10.00	0.065	15.25	0.149	26.06	-	-	0.274	48.96	-	-	0.163	0.06	30.09	9.94
	11.25	0.060	14.01	0.069	12.10	-	-	0.183	32.68	-	-	0.104	0.04	19.60	6.56
	12.50	0.067	15.56	0.088	15.42	-	-	0.130	23.26	-	-	0.095	0.02	18.08	2.59
	13.75	0.057	13.31	0.066	11.58	-	-	0.100	17.89	-	-	0.074	0.01	14.26	1.88
	15.00	0.050	11.60	0.050	8.73	-	-	0.119	21.29	-	-	0.073	0.02	13.87	3.80
	16.25	0.050	11.67	0.052	9.02	-	-	0.075	13.36	-	-	0.059	0.01	11.35	1.26
	17.50	0.050	11.67	0.053	9.19	-	-	0.073	13.00	-	-	0.058	0.01	11.29	1.12
	18.75	0.049	11.52	0.049	8.49	-	-	0.066	11.87	-	-	0.055	0.01	10.63	1.07
	20.00	-	-	0.052	9.02	-	-	0.063	11.21	0.048	6.91	0.054	0.00	9.05	1.24

Table D2 – Neutral red assay data, of 5 independent experiments, for sample C after 48h exposure.

Sample	Concentration (ul/ml)	1st Assay		2nd Assay		3rd Assay		4th Assay		5th Assay		Mean		Mean	
		Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	SE	$\%_{Viability}$	SE
C	0.00	0.428	100.00	0.573	100.00	0.576	100.00	0.559	100.00	0.690	100.00	0.565	0.05	100.00	0.00
	0.10	-	-	0.631	110.18	0.624	108.39	0.548	97.97	-	-	0.601	0.03	105.51	3.81
	0.20	-	-	0.555	96.86	0.619	107.41	0.540	96.60	-	-	0.571	0.02	100.29	3.56
	0.40	-	-	0.535	93.37	0.626	108.74	0.489	87.48	-	-	0.550	0.04	96.53	6.34
	0.60	-	-	0.673	117.39	0.627	108.91	0.615	109.96	-	-	0.638	0.02	112.09	2.67
	0.80	-	-	0.648	113.15	0.643	111.69	0.632	113.00	-	-	0.641	0.00	112.61	0.46
	1.00	-	-	0.627	109.48	0.575	99.77	0.660	118.01	-	-	0.621	0.02	109.09	5.27
	1.25	0.510	119.14	0.593	103.55	-	-	0.633	113.30	-	-	0.579	0.04	112.00	4.55
	1.50	-	-	-	-	0.619	107.41	0.582	104.05	0.704	102.08	0.635	0.04	104.51	1.56
	2.00	-	-	-	-	0.627	108.80	0.581	103.88	0.613	88.84	0.607	0.01	100.50	6.00
	2.50	0.536	125.06	0.531	92.61	0.594	103.07	-	-	-	-	0.553	0.02	106.91	9.56
	3.00	-	-	-	-	0.634	110.07	0.607	108.53	0.734	106.38	0.658	0.04	108.33	1.07
	3.50	-	-	-	-	0.650	112.91	0.548	97.97	0.690	100.00	0.629	0.04	103.63	4.68
	3.75	0.352	82.24	0.522	91.04	-	-	0.625	111.75	-	-	0.500	0.08	95.01	8.75
	4.00	-	-	-	-	0.650	112.85	0.569	101.79	0.661	95.84	0.627	0.03	103.49	4.98
	4.50	-	-	-	-	0.631	109.61	0.570	101.97	0.651	94.44	0.618	0.02	102.01	4.38
	5.00	0.413	96.42	0.623	108.78	0.635	110.30	-	-	-	-	0.557	0.07	105.17	4.40
	6.25	0.432	100.93	0.591	103.08	-	-	0.564	100.95	-	-	0.529	0.05	101.66	0.71
	7.50	0.395	92.22	0.484	84.53	-	-	0.649	116.16	-	-	0.510	0.07	97.63	9.53
	8.75	0.367	85.76	0.501	87.49	-	-	0.550	98.39	-	-	0.473	0.05	90.55	3.95
	10.00	0.377	88.02	0.455	79.46	-	-	0.597	106.80	-	-	0.476	0.06	91.43	8.07
	11.25	0.354	82.65	0.440	76.79	-	-	0.501	89.68	-	-	0.432	0.04	83.04	3.73
	12.50	0.397	92.61	0.522	91.10	-	-	0.621	111.09	-	-	0.513	0.06	98.27	6.43
	13.75	0.402	93.93	0.413	72.08	-	-	0.493	88.13	-	-	0.436	0.03	84.71	6.54
	15.00	0.333	77.74	0.486	84.76	-	-	0.516	92.37	-	-	0.445	0.06	84.96	4.22
	16.25	0.360	83.97	0.428	74.75	-	-	0.470	84.02	-	-	0.419	0.03	80.91	3.08
	17.50	0.344	80.31	0.436	76.15	-	-	0.621	111.09	-	-	0.467	0.08	89.18	11.02
	18.75	0.335	78.21	0.281	49.10	-	-	0.511	91.41	-	-	0.376	0.07	72.91	12.50
	20.00	-	-	0.357	62.25	-	-	0.459	82.17	0.517	75.01	0.444	0.05	73.14	5.83

Table D3 – Neutral red assay data, of 3 independent experiments, for sample E after 48h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
E	0.00	0.970	100.00	0.799	100.00	0.596	100.00	0.788	0.11	100.00	0.00
	0.10	0.815	84.05	0.810	101.42	0.751	126.13	0.792	0.02	103.87	12.21
	0.50	0.925	95.43	0.840	105.22	0.773	129.83	0.846	0.04	110.16	10.23
	1.00	0.868	89.52	0.762	95.37	0.718	120.59	0.783	0.04	101.83	9.53
	1.25	0.823	84.84	0.848	106.22	0.679	113.99	0.783	0.05	101.68	8.72
	2.50	0.792	81.71	0.770	96.45	0.718	120.59	0.760	0.02	99.59	11.33
	3.75	0.902	93.06	0.802	100.42	0.803	134.86	0.836	0.03	109.45	12.89
	5.00	0.863	88.97	0.705	88.27	0.769	129.16	0.779	0.05	102.13	13.51
	6.35	0.787	81.16	0.726	90.94	0.609	102.18	0.707	0.05	91.43	6.07
	7.50	0.674	69.54	0.633	79.30	0.631	105.99	0.646	0.01	84.94	10.89
	8.75	0.814	83.98	0.704	88.15	0.582	97.71	0.700	0.07	89.94	4.06
	10.00	0.758	78.21	0.638	79.84	0.624	104.70	0.673	0.04	87.58	8.57
	12.50	0.710	73.26	0.536	67.11	0.498	83.55	0.581	0.07	74.64	4.79
	15.00	0.541	55.83	0.452	56.64	0.419	70.34	0.471	0.04	60.93	4.71
	17.50	0.376	38.74	0.526	65.90	0.252	42.25	0.385	0.08	48.96	8.53
	20.00	0.345	35.61	0.513	64.27	0.215	36.15	0.358	0.09	45.35	9.47

Table D4 – Neutral red assay data, of 3 independent experiments, for sample P after 48h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
P	0.00	0.970	100.00	0.799	100.00	0.596	100.00	0.788	0.11	100.00	0.00
	0.10	0.738	76.14	0.916	114.65	0.681	114.27	0.778	0.07	101.69	12.77
	0.50	0.777	80.17	0.796	99.71	0.629	105.60	0.734	0.05	95.16	7.69
	1.00	0.815	84.08	0.882	110.43	0.548	92.05	0.749	0.10	95.52	7.80
	1.25	0.788	81.30	0.862	107.97	0.651	109.29	0.767	0.06	99.52	9.12
	2.50	0.663	68.37	0.854	106.89	0.658	110.52	0.725	0.06	95.26	13.48
	3.75	0.751	77.48	0.703	88.02	0.715	119.98	0.723	0.01	95.16	12.78
	5.00	0.653	67.31	0.716	89.69	0.384	64.52	0.584	0.10	73.84	7.97
	6.35	0.552	56.89	0.629	78.71	0.362	60.83	0.514	0.08	65.48	6.71
	7.50	0.372	38.40	0.567	71.04	0.524	87.91	0.488	0.06	65.78	14.53
	8.75	0.552	56.96	0.357	44.70	0.418	70.17	0.442	0.06	57.28	7.36
	10.00	0.247	25.51	0.305	38.19	0.405	68.05	0.319	0.05	43.91	12.61
	12.50	0.185	19.04	0.238	29.80	0.239	40.18	0.221	0.02	29.67	6.10
	15.00	0.144	14.85	0.183	22.87	0.134	22.50	0.154	0.01	20.07	2.61
	17.50	0.150	15.50	0.136	17.07	0.084	14.16	0.124	0.02	15.58	0.84
	20.00	0.103	10.59	0.101	12.69	0.131	21.99	0.112	0.01	15.09	3.50

Table D5 – Neutral red assay data, of 3 independent experiments, for sample A after 48h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
A	0.00	0.970	100.00	0.799	100.00	0.596	100.00	0.788	0.11	100.00	0.00
	0.10	0.838	86.39	0.906	113.48	0.756	126.92	0.833	0.04	108.93	11.92
	0.50	0.833	85.94	0.893	111.81	0.764	128.20	0.830	0.04	108.65	12.30
	1.00	0.875	90.24	0.819	102.59	0.740	124.17	0.811	0.04	105.67	9.92
	1.25	0.855	88.21	0.838	104.92	0.683	114.72	0.792	0.05	102.62	7.74
	2.50	0.852	87.83	0.822	102.96	0.724	121.60	0.799	0.04	104.13	9.77
	3.75	0.816	84.12	0.819	102.50	0.708	118.91	0.781	0.04	101.85	10.05
	5.00	0.806	83.09	0.759	95.03	0.682	114.44	0.749	0.04	97.52	9.14
	6.35	0.762	78.55	0.747	93.53	0.624	104.76	0.711	0.04	92.28	7.59
	7.50	0.775	79.96	0.704	88.15	0.600	100.67	0.693	0.05	89.59	6.02
	8.75	0.661	68.13	0.684	85.64	0.643	108.00	0.663	0.01	87.26	11.54
	10.00	0.774	79.82	0.630	78.92	0.681	114.27	0.695	0.04	91.00	11.64
	12.50	0.556	57.30	0.599	75.00	0.378	63.51	0.511	0.07	65.27	5.18
	15.00	0.506	52.18	0.588	73.58	0.322	54.11	0.472	0.08	59.96	6.83
	17.50	0.402	41.42	0.507	63.44	0.366	61.50	0.425	0.04	55.45	7.04
	20.00	0.534	55.04	0.452	56.55	0.553	92.78	0.513	0.03	68.12	12.34

Table D6 – Neutral red assay data, of 5 independent experiments, for sample M after 48h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	4th Assay Abs_{540nm}	4th Assay $\%_{Viability}$	5th Assay Abs_{540nm}	5th Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
M	0.00	0.428	100.00	0.573	100.00	0.576	100.00	0.559	100.00	0.690	100.00	0.565	0.05	100.00	0.00
	0.10	-	-	0.588	102.62	0.619	107.47	0.541	96.78	-	-	0.583	0.02	102.29	3.09
	0.20	-	-	0.508	88.71	0.628	109.03	0.559	100.06	-	-	0.565	0.03	99.27	5.88
	0.40	-	-	0.594	103.72	0.614	106.66	0.583	104.29	-	-	0.597	0.01	104.89	0.90
	0.60	-	-	0.542	94.53	0.629	109.20	0.618	110.49	-	-	0.596	0.03	104.74	5.12
	0.80	-	-	0.609	106.22	0.632	109.66	0.589	105.31	-	-	0.610	0.01	107.07	1.33
	1.00	-	-	0.535	93.43	0.540	93.81	0.612	109.42	-	-	0.562	0.02	98.89	5.27
	1.25	0.494	115.33	0.486	84.87	-	-	0.636	113.77	-	-	0.539	0.05	104.66	9.90
	1.50	-	-	-	-	0.563	97.74	0.620	110.91	0.681	98.74	0.621	0.03	102.47	4.23
	2.00	-	-	-	-	0.599	104.05	0.532	95.11	0.598	86.71	0.576	0.02	95.29	5.01
	2.50	0.402	93.85	0.583	101.69	0.505	87.73	-	-	-	-	0.497	0.05	94.42	4.04
	3.00	-	-	-	-	0.544	94.50	0.584	104.47	0.546	79.17	0.558	0.01	92.71	7.36
	3.50	-	-	-	-	0.480	83.28	0.567	101.37	0.526	76.22	0.524	0.03	86.96	7.49
	3.75	0.294	68.72	0.397	69.28	-	-	0.552	98.69	-	-	0.414	0.07	78.90	9.90
	4.00	-	-	-	-	0.461	80.09	0.502	89.80	0.568	82.41	0.511	0.03	84.10	2.93
	4.50	-	-	-	-	0.520	90.34	0.432	77.22	0.547	79.31	0.500	0.03	82.29	4.07
	5.00	0.280	65.29	0.371	64.75	0.451	78.30	-	-	-	-	0.367	0.05	69.45	4.43
	6.25	0.145	33.93	0.400	69.75	-	-	0.431	77.16	-	-	0.325	0.09	60.28	13.35
	7.50	0.079	18.44	0.234	40.78	-	-	0.490	87.60	-	-	0.267	0.12	48.94	20.38
	8.75	0.090	21.01	0.117	20.48	-	-	0.393	70.30	-	-	0.200	0.10	37.26	16.52
	10.00	0.072	16.73	0.139	24.20	-	-	0.267	47.70	-	-	0.159	0.06	29.55	9.33
	11.25	0.054	12.61	0.078	13.55	-	-	0.134	24.03	-	-	0.089	0.02	16.73	3.66
	12.50	0.052	12.06	0.051	8.90	-	-	0.095	16.94	-	-	0.066	0.01	12.63	2.34
	13.75	0.049	11.36	0.050	8.67	-	-	0.080	14.37	-	-	0.060	0.01	11.47	1.65
	15.00	0.045	10.43	0.048	8.32	-	-	0.094	16.88	-	-	0.062	0.02	11.87	2.57
	16.25	0.049	11.52	0.051	8.90	-	-	0.063	11.33	-	-	0.055	0.00	10.58	0.84
	17.50	0.048	11.21	0.049	8.55	-	-	0.056	10.02	-	-	0.051	0.00	9.93	0.77
	18.75	0.049	11.44	0.051	8.96	-	-	0.047	8.47	-	-	0.049	0.00	9.62	0.92
	20.00	-	-	0.049	8.61	-	-	0.059	10.49	0.050	7.30	0.053	0.00	8.80	0.93

Table D7 – Neutral red assay data, of 3 independent experiments, for sample Mf after 48h exposure.

Sample	Concentration (µl/ml)	1st Assay Abs_{540nm}	1st Assay $\%_{Viability}$	2nd Assay Abs_{540nm}	2nd Assay $\%_{Viability}$	3rd Assay Abs_{540nm}	3rd Assay $\%_{Viability}$	Mean Abs_{540nm}	SE	Mean $\%_{Viability}$	SE
Mf	0.00	0.970	100.00	0.799	100.00	0.596	100.00	0.788	0.11	100.00	0.00
	0.10	0.971	100.17	0.885	110.77	0.614	103.08	0.823	0.11	104.67	3.16
	0.50	0.888	91.54	0.790	98.87	0.722	121.15	0.800	0.05	103.86	8.90
	1.00	0.866	89.31	0.873	109.31	0.746	125.29	0.828	0.04	107.97	10.41
	1.25	0.860	88.66	0.909	113.77	0.763	128.15	0.844	0.04	110.19	11.54
	2.50	0.945	97.49	0.797	99.75	0.615	103.25	0.786	0.10	100.16	1.67
	3.75	0.813	83.81	0.814	101.92	0.718	120.48	0.781	0.03	102.07	10.59
	5.00	0.833	85.91	0.791	99.00	0.700	117.52	0.775	0.04	100.81	9.17
	6.35	0.779	80.37	0.813	101.84	0.722	121.15	0.771	0.03	101.12	11.78
	7.50	0.896	92.40	0.795	99.54	0.620	104.14	0.770	0.08	98.69	3.41
	8.75	0.762	78.62	0.724	90.65	0.669	112.37	0.719	0.03	93.88	9.88
	10.00	0.759	78.31	0.657	82.30	0.640	107.44	0.686	0.04	89.35	9.12
	12.50	0.676	69.75	0.655	82.01	0.554	92.95	0.628	0.04	81.57	6.70
	15.00	0.780	80.44	0.627	78.51	0.607	101.90	0.671	0.05	86.95	7.50
	17.50	0.649	66.90	0.537	67.24	0.500	83.94	0.562	0.04	72.69	5.63
	20.00	0.555	57.27	0.501	62.69	0.494	82.99	0.517	0.02	67.65	7.83

Table D8 – Neutral red assay data, of 5 independent experiments, for DMSO after 48h exposure.

Sample	Concentration (µl/ml)	1st Assay		2nd Assay		3rd Assay		4th Assay		5th Assay		Mean		Mean	
		Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	$\%_{Viability}$	Abs_{540nm}	SE	$\%_{Viability}$	SE
DMSO	0.00	0.428	100.00	0.573	100.00	0.576	100.00	0.559	100.00	0.690	100.00	0.565	0.05	100.00	0.00
	0.10	-	-	0.541	94.36	0.602	104.46	0.634	113.36	-	-	0.592	0.03	104.06	5.49
	0.50	-	-	0.492	85.92	0.659	114.35	0.609	109.00	-	-	0.587	0.05	103.09	8.72
	1.00	-	-	0.523	91.33	0.587	101.97	0.611	109.36	-	-	0.574	0.03	100.89	5.23
	5.00	-	-	-	-	0.530	92.01	0.657	117.59	0.655	95.02	0.614	0.04	101.54	8.07
	10.00	0.289	67.55	0.611	106.57	-	-	0.559	99.94	-	-	0.486	0.10	91.35	12.06
	20.00	0.246	57.51	-	-	-	-	0.452	80.86	0.439	63.70	0.379	0.07	67.36	6.98

ANNEX E – Experimental data obtained in the standard alkaline comet assay following a 48h exposure period to sediment extracts.

Table E1 – Standard alkaline comet assay data, of 3 independent experiments, for sample F.

	Concentration			DNA in Tail (%)		
Sample	(µl/ml)	1st Assay	2nd Assay	3rd Assay	Mean	SE
DMSO	0.00	2.30	1.82	2.48	2.20	0.20
	5.00	1.91	2.95	2.59	2.48	0.30
EMS	**	39.31	37.62	34.56	37.17	1.39
F	0.50	2.27	1.81	1.40	1.82	0.25
	1.00	2.57	1.65	2.85	2.35	0.36
	1.25	1.71	2.51	3.54	2.59	0.53
	2.00	2.76	2.16	4.21	3.04	0.61
	2.50	7.18	3.37	2.74	4.43	1.39
	4.00	2.65	3.10	2.63	2.79	0.15
	5.00	17.69	1.83	4.93	8.15	4.85
	Concentration			Tail Lenght (µm)		
Sample	(µl/ml)	1st Assay	2nd Assay	3rd Assay	Mean	SE
DMSO	0.00	4.66	3.53	5.97	4.72	0.70
	5.00	3.69	6.68	5.64	5.34	0.88
EMS	**	47.66	44.80	42.05	44.84	1.62
F	0.50	5.26	2.79	2.94	3.67	0.80
	1.00	3.42	1.98	7.23	4.21	1.56
	1.25	4.96	3.69	10.84	6.49	2.20
	2.00	6.98	3.02	12.62	7.54	2.79
	2.50	9.67	6.77	5.08	7.18	1.34
	4.00	6.94	4.09	5.60	5.54	0.82
	5.00	26.15	3.74	16.39	15.43	6.49
	Concentration			Tail Moment (%)		
Sample	(µl/ml)	1st Assay	2nd Assay	3rd Assay	Mean	SE
DMSO	0.00	0.19	0.13	0.27	0.20	0.04
	5.00	0.15	0.30	0.28	0.24	0.05
EMS	**	19.33	17.37	14.96	17.22	1.26
F	0.50	0.22	0.13	0.10	0.15	0.04
	1.00	0.18	0.09	0.34	0.20	0.07
	1.25	0.20	0.22	0.52	0.31	0.10
	2.00	0.34	0.12	0.73	0.40	0.18
	2.50	1.33	0.39	0.28	0.67	0.33
	4.00	0.30	0.26	0.31	0.29	0.02
	5.00	6.88	0.15	0.97	2.67	2.12

** EMS concentration 30mM.

Table E2 – Standard alkaline comet assay data, of 3 independent experiments, for sample C.

Sample	Concentration (µl/ml)	DNA in Tail (%) 1st Assay	2nd Assay	3rd Assay	Mean	SE
DMSO	0.00	2.30	1.82	2.48	2.20	0.20
	5.00	1.91	2.95	2.59	2.48	0.30
EMS	**	39.31	37.62	34.56	37.17	1.39
C	0.50	3.54	2.68	2.30	2.84	0.37
	1.00	2.33	2.29	2.62	2.41	0.10
	1.25	2.00	2.61	2.99	2.53	0.29
	2.00	2.83	2.57	1.84	2.41	0.30
	2.50	2.80	3.32	2.52	2.88	0.23
	4.00	1.50	2.23	2.03	1.92	0.22
	5.00	2.18	2.66	2.55	2.46	0.14
Sample	Concentration (µl/ml)	Tail Lenght (µm) 1st Assay	2nd Assay	3rd Assay	Mean	SE
DMSO	0.00	4.66	3.53	5.97	4.72	0.70
	5.00	3.69	6.68	5.64	5.34	0.88
EMS	**	47.66	44.80	42.05	44.84	1.62
C	0.50	8.16	5.79	3.11	5.69	1.46
	1.00	3.96	5.05	5.52	4.84	0.46
	1.25	4.61	5.65	5.90	5.39	0.40
	2.00	7.26	5.64	2.34	5.08	1.45
	2.50	6.86	10.30	7.07	8.08	1.11
	4.00	2.90	5.57	3.36	3.94	0.82
	5.00	6.93	6.97	5.20	6.36	0.58
Sample	Concentration (µl/ml)	Tail Moment (%) 1st Assay	2nd Assay	3rd Assay	Mean	SE
DMSO	0.00	0.19	0.13	0.27	0.20	0.04
	5.00	0.15	0.30	0.28	0.24	0.05
EMS	**	19.33	17.37	14.96	17.22	1.26
C	0.50	0.43	0.32	0.15	0.30	0.08
	1.00	0.19	0.22	0.27	0.23	0.02
	1.25	0.19	0.24	0.33	0.26	0.04
	2.00	0.31	0.24	0.09	0.21	0.06
	2.50	0.28	0.49	0.25	0.34	0.08
	4.00	0.13	0.21	0.18	0.17	0.02
	5.00	0.28	0.29	0.23	0.27	0.02

** EMS concentration 30mM.

Table E3 – Standard alkaline comet assay data, of 3 independent experiments, for sample M.

Sample	Concentration (µl/ml)	DNA in Tail (%) 1st Assay	2nd Assay	3rd Assay	Mean	SE
DMSO	0.00	2.30	1.82	2.48	2.20	0.20
	5.00	1.91	2.95	2.59	2.48	0.30
EMS	**	39.31	37.62	34.56	37.17	1.39
M	0.50	3.60	3.11	2.50	3.07	0.32
	1.00	2.06	2.35	2.02	2.14	0.10
	1.25	2.40	2.31	2.16	2.29	0.07
	2.00	2.40	2.41	1.70	2.17	0.23
	2.50	4.37	2.89	1.86	3.04	0.73
	4.00	2.11	2.06	2.44	2.20	0.12
	5.00	9.30	2.01	2.58	4.63	2.34
Sample	Concentration (µl/ml)	Tail Lenght (µm) 1st Assay	2nd Assay	3rd Assay	Mean	SE
DMSO	0.00	4.66	3.53	5.97	4.72	0.70
	5.00	3.69	6.68	5.64	5.34	0.88
EMS	**	47.66	44.80	42.05	44.84	1.62
M	0.50	9.96	5.20	5.93	7.03	1.48
	1.00	3.66	4.32	4.50	4.16	0.26
	1.25	4.63	3.68	4.34	4.22	0.28
	2.00	3.74	4.18	4.42	4.11	0.20
	2.50	7.27	8.21	2.30	5.93	1.83
	4.00	2.52	3.97	6.90	4.46	1.29
	5.00	11.92	3.53	4.89	6.78	2.60
Sample	Concentration (µl/ml)	Tail Moment (%) 1st Assay	2nd Assay	3rd Assay	Mean	SE
DMSO	0.00	0.19	0.13	0.27	0.20	0.04
	5.00	0.15	0.30	0.28	0.24	0.05
EMS	**	19.33	17.37	14.96	17.22	1.26
M	0.50	0.51	0.29	0.28	0.36	0.08
	1.00	0.15	0.22	0.18	0.18	0.02
	1.25	0.20	0.18	0.18	0.18	0.01
	2.00	0.17	0.16	0.14	0.16	0.01
	2.50	0.52	0.36	0.12	0.33	0.12
	4.00	0.14	0.16	0.29	0.19	0.05
	5.00	1.68	0.14	0.25	0.69	0.49

** EMS concentration 30mM.

ANNEX F – Experimental data obtained in the alkaline comet assay coupled with FPG, following a 48h exposure period.

Table F1 – Alkaline comet assay with FPG data, of 3 independent experiments, for sample F.

DNA in Tail (%)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE	FPG sensitive sites	SE
DMSO	0.00	1.84	3.09	2.70	2.78	3.35	3.80	2.63	0.43	3.22	0.30	0.59	0.34
	5.00	1.75	3.64	2.36	2.67	3.00	5.97	2.37	0.36	4.09	0.98	1.72	0.77
	20.00	3.20	5.10	2.98	2.59	2.37	3.49	2.85	0.25	3.73	0.73	0.88	0.67
H_2O_2	**	51.79	66.77	45.91	63.68	53.52	65.98	50.41	2.30	65.48	0.93	15.07	1.54
F	1.00	2.47	6.43	2.89	5.57	3.94	4.69	3.10	0.44	5.56	0.50	2.46	0.93
	2.50	3.47	10.81	3.38	8.06	3.61	8.59	3.49	0.07	9.15	0.84	5.67	0.84
	5.00	10.05	12.29	4.88	16.98	8.99	12.26	7.98	1.58	13.84	1.57	5.87	3.13

Tail Lenght (µm)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE
DMSO	0.00	3.13	4.68	4.59	5.33	6.12	4.71	4.61	0.86	4.91	0.21
	5.00	2.47	5.27	4.47	3.54	8.27	8.79	5.07	1.70	5.87	1.54
	20.00	5.54	8.06	4.35	3.82	4.78	5.39	4.89	0.35	5.76	1.24
H_2O_2	**	44.41	68.14	39.21	57.77	47.99	54.51	43.87	2.55	60.14	4.11
F	1.00	2.75	8.24	5.53	8.41	7.00	7.13	5.10	1.25	7.93	0.40
	2.50	4.51	12.53	4.89	9.32	6.23	13.09	5.21	0.52	11.65	1.17
	5.00	11.05	14.32	7.18	21.79	15.57	18.60	11.27	2.43	18.24	2.16

Tail Moment (%)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE
DMSO	0.00	0.12	0.20	0.24	0.23	0.34	0.27	0.23	0.06	0.23	0.02
	5.00	0.11	0.29	0.18	0.15	0.36	0.64	0.22	0.08	0.36	0.15
	20.00	0.30	0.53	0.21	0.18	0.20	0.27	0.24	0.03	0.33	0.11
H_2O_2	**	25.92	49.15	20.49	38.97	28.51	37.55	24.98	2.36	41.89	3.65
F	1.00	0.16	0.72	0.29	0.64	0.47	0.53	0.31	0.09	0.63	0.06
	2.50	0.32	1.79	0.29	1.04	0.38	1.52	0.33	0.03	1.45	0.22
	5.00	1.88	3.03	0.48	4.50	1.71	2.56	1.36	0.44	3.36	0.59

** H_2O_2 concentration at 200µM.

Table F2 – Alkaline comet assay with FPG data, of 3 independent experiments, for sample C.

DNA in Tail (%)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE	FPG sensitive sites	SE
DMSO	0.00	1.84	3.09	2.70	2.78	3.35	3.80	2.63	0.43	3.22	0.30	0.59	0.34
	5.00	1.75	3.64	2.36	2.67	3.00	5.97	2.37	0.36	4.09	0.98	1.72	0.77
	20.00	3.20	5.10	2.98	2.59	2.37	3.49	2.85	0.25	3.73	0.73	0.88	0.67
H_2O_2	**	51.79	66.77	45.91	63.68	53.52	65.98	50.41	2.30	65.48	0.93	15.07	1.54
C	1.00	2.01	2.70	2.49	3.62	3.78	6.67	2.76	0.53	4.33	1.20	1.57	0.67
	2.50	2.73	3.74	3.02	8.75	3.86	5.26	3.21	0.34	5.92	1.48	2.71	1.51
	5.00	2.15	3.70	3.61	5.07	4.36	4.57	3.37	0.65	4.45	0.40	1.07	0.43
	10.00	2.35	4.84	3.49	4.58	4.69	5.87	3.51	0.68	5.09	0.39	1.58	0.46
	15.00	3.54	3.36	3.33	4.37	3.40	4.77	3.42	0.06	4.17	0.42	0.75	0.47
	20.00	2.86	5.09	3.09	8.22	4.37	5.79	3.44	0.47	6.37	0.95	2.93	1.12

Tail Lenght (µm)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE
DMSO	0.00	3.13	4.68	4.59	5.33	6.12	4.71	4.61	0.86	4.91	0.21
	5.00	2.47	5.27	4.47	3.54	8.27	8.79	5.07	1.70	5.87	1.54
	20.00	5.54	8.06	4.35	3.82	4.78	5.39	4.89	0.35	5.76	1.24
H_2O_2	**	44.41	68.14	39.21	57.77	47.99	54.51	43.87	2.55	60.14	4.11
C	1.00	2.46	5.83	4.87	5.25	5.66	12.39	4.33	0.96	7.82	2.29
	2.50	6.75	6.98	4.40	11.33	6.35	9.37	5.83	0.73	9.23	1.26
	5.00	2.92	4.80	8.04	6.16	11.07	10.47	7.35	2.38	7.14	1.71
	10.00	3.24	6.97	3.79	7.36	11.62	11.26	6.22	2.71	8.53	1.37
	15.00	5.27	6.00	6.51	7.42	2.65	11.53	4.81	1.14	8.32	1.66
	20.00	4.88	6.62	3.30	8.96	8.58	7.92	5.58	1.56	7.83	0.68

Table F2 – (cont.)

		Tail Moment (%)									
	Concentration	1st Assay		2nd Assay		3rd Assay		Mean		Mean	
Sample	(µl/ml)	without FPG	with FPG	without FPG	with FPG	without FPG	with FPG	without FPG	SE	with FPG	SE
DMSO	0.00	0.12	0.20	0.24	0.23	0.34	0.27	0.23	0.06	0.23	0.02
	5.00	0.11	0.29	0.18	0.15	0.36	0.64	0.22	0.08	0.36	0.15
	20.00	0.30	0.53	0.21	0.18	0.20	0.27	0.24	0.03	0.33	0.11
H_2O_2	**	25.92	49.15	20.49	38.97	28.51	37.55	24.98	2.36	41.89	3.65
C	1.00	0.11	0.24	0.23	0.27	0.33	1.00	0.22	0.07	0.51	0.25
	2.50	0.31	0.42	0.27	1.42	0.39	0.68	0.33	0.04	0.84	0.30
	5.00	0.14	0.30	0.40	0.47	0.71	0.62	0.42	0.16	0.46	0.09
	10.00	0.16	0.45	0.24	0.47	0.87	0.81	0.42	0.22	0.58	0.12
	15.00	0.30	0.29	0.34	0.47	0.18	0.71	0.28	0.05	0.49	0.12
	20.00	0.24	0.47	0.21	0.95	0.51	0.67	0.32	0.10	0.70	0.14

** H_2O_2 concentration at 200µM.

Table F3 – Alkaline comet assay with FPG data, of 3 independent experiments, for sample E.

		DNA in Tail (%)											
	Concentration	1st Assay		2nd Assay		3rd Assay		Mean		Mean		FPG sensitive	
Sample	(µl/ml)	without FPG	with FPG	without FPG	with FPG	without FPG	with FPG	without FPG	SE	with FPG	SE	sites	SE
DMSO	5.00	3.12	4.40	3.69	5.51	4.17	4.29	3.66	0.30	4.73	0.39	1.07	0.50
	20.00	4.29	6.06	3.68	5.67	3.31	4.85	3.76	0.29	5.53	0.36	1.77	0.13
H_2O_2	**	59.00	66.15	53.97	65.65	48.54	63.96	53.84	3.02	65.25	0.66	11.42	2.39
E	1.00	2.78	4.42	3.96	3.91	4.02	4.36	3.59	0.41	4.23	0.16	0.64	0.51
	2.50	3.84	6.98	3.98	5.81	2.92	4.98	3.58	0.33	5.92	0.58	2.34	0.40
	5.00	4.27	8.01	3.00	8.68	3.43	3.31	3.57	0.37	6.67	1.69	3.10	1.71
	10.00	4.55	13.62	4.14	21.46	3.78	20.07	4.16	0.22	18.39	2.41	14.23	2.60
	20.00	6.03	26.34	6.32	31.67	7.80	30.86	6.72	0.55	29.63	1.66	22.91	1.46

		Tail Lenght (µm)									
	Concentration	1st Assay		2nd Assay		3rd Assay		Mean		Mean	
Sample	(µl/ml)	without FPG	with FPG	without FPG	with FPG	without FPG	with FPG	without FPG	SE	with FPG	SE
DMSO	5.00	5.13	6.88	6.98	7.47	7.78	8.07	6.63	0.79	7.47	0.34
	20.00	8.04	12.79	4.89	10.75	6.64	7.44	6.53	0.91	10.32	1.56
H_2O_2	**	65.73	64.59	52.65	64.08	44.17	62.42	54.18	6.27	63.70	0.65
E	1.00	6.79	6.24	5.58	6.55	4.40	6.75	5.59	0.69	6.51	0.15
	2.50	7.38	11.02	6.35	7.29	4.52	9.02	6.08	0.84	9.11	1.08
	5.00	7.70	12.89	4.10	10.43	6.75	4.76	6.19	1.08	9.36	2.41
	10.00	6.29	16.15	6.72	31.32	7.18	27.72	6.73	0.26	25.06	4.57
	20.00	8.67	32.35	7.39	41.11	13.03	41.89	9.70	1.71	38.45	3.06

		Tail Moment (%)									
	Concentration	1st Assay		2nd Assay		3rd Assay		Mean		Mean	
Sample	(µl/ml)	without FPG	with FPG	without FPG	with FPG	without FPG	with FPG	without FPG	SE	with FPG	SE
DMSO	5.00	0.29	0.44	0.41	0.67	0.48	0.46	0.39	0.06	0.52	0.07
	20.00	0.58	1.01	0.30	0.78	0.41	0.60	0.43	0.08	0.80	0.12
H_2O_2	**	42.02	45.95	31.00	46.01	23.61	42.35	32.21	5.35	44.77	1.21
E	1.00	0.30	0.39	0.38	0.39	0.34	0.44	0.34	0.02	0.40	0.02
	2.50	0.47	0.98	0.41	0.58	0.24	0.56	0.38	0.07	0.70	0.14
	5.00	0.51	1.20	0.23	1.09	0.33	0.30	0.36	0.08	0.86	0.28
	10.00	0.47	2.69	0.43	7.26	0.43	6.70	0.44	0.01	5.55	1.44
	20.00	0.76	9.12	0.70	14.37	1.23	14.56	0.90	0.17	12.68	1.78

** H_2O_2 concentration at 200µM.

Table F4 – Alkaline comet assay with FPG data, of 3 independent experiments, for sample P.

DNA in Tail (%)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE	FPG sensitive sites	SE
DMSO	5.00	3.12	4.40	3.69	5.51	4.17	4.29	3.66	0.30	4.73	0.39	1.07	0.50
	20.00	4.29	6.06	3.68	5.67	3.31	4.85	3.76	0.29	5.53	0.36	1.77	0.13
H_2O_2	**	59.00	66.15	53.97	65.65	48.54	63.96	53.84	3.02	65.25	0.66	11.42	2.39
P	1.00	2.43	5.99	3.94	5.57	3.52	5.78	3.30	0.45	5.78	0.12	2.49	0.57
	2.50	4.06	5.87	4.78	8.40	3.90	8.19	4.25	0.27	7.49	0.81	3.24	0.74
	5.00	6.04	15.41	2.75	18.35	4.45	11.55	4.42	0.95	15.10	1.97	10.69	2.54
	10.00	9.98	19.19	10.66	18.85	10.32	22.60	10.32	0.19	20.22	1.20	9.90	1.23

Tail Lenght (µm)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE
DMSO	5.00	5.13	6.88	6.98	7.47	7.78	8.07	6.63	0.79	7.47	0.34
	20.00	8.04	12.79	4.89	10.75	6.64	7.44	6.53	0.91	10.32	1.56
H_2O_2	**	65.73	64.59	52.65	64.08	44.17	62.42	54.18	6.27	63.70	0.65
P	1.00	4.83	7.65	6.79	7.36	6.21	7.59	5.94	0.58	7.53	0.09
	2.50	5.37	9.89	8.99	9.82	6.81	9.71	7.06	1.05	9.81	0.05
	5.00	8.32	18.97	4.62	20.25	5.38	12.80	6.11	1.13	17.34	2.30
	10.00	13.77	25.91	14.50	29.26	13.87	32.74	14.04	0.23	29.30	1.97

Tail Moment (%)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE
DMSO	5.00	0.29	0.44	0.41	0.67	0.48	0.46	0.39	0.06	0.52	0.07
	20.00	0.58	1.01	0.30	0.78	0.41	0.60	0.43	0.08	0.80	0.12
H_2O_2	**	42.02	45.95	31.00	46.01	23.61	42.35	32.21	5.35	44.77	1.21
P	1.00	0.20	0.61	0.46	0.58	0.40	0.63	0.35	0.08	0.61	0.01
	2.50	0.35	0.74	0.60	1.09	0.41	0.96	0.46	0.08	0.93	0.10
	5.00	0.70	3.70	0.27	4.63	0.42	1.74	0.46	0.13	3.36	0.85
	10.00	1.84	5.69	2.07	6.20	1.97	8.47	1.96	0.06	6.79	0.85

** H_2O_2 concentration at 200µM.

Table F5 – Alkaline comet assay with FPG data, of 3 independent experiments, for sample A.

DNA in Tail (%)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE	FPG sensitive sites	SE
DMSO	5.00	3.12	4.40	3.69	5.51	4.17	4.29	3.66	0.30	4.73	0.39	1.07	0.50
	20.00	4.29	6.06	3.68	5.67	3.31	4.85	3.76	0.29	5.53	0.36	1.77	0.13
H_2O_2	**	59.00	66.15	53.97	65.65	48.54	63.96	53.84	3.02	65.25	0.66	11.42	2.39
A	1.00	3.68	5.26	4.26	4.50	3.58	5.53	3.84	0.21	5.10	0.31	1.26	0.52
	2.50	4.86	5.82	3.99	5.92	4.10	4.86	4.31	0.27	5.53	0.34	1.22	0.36
	5.00	3.58	5.24	4.44	6.98	4.47	5.56	4.16	0.29	5.93	0.54	1.76	0.42
	10.00	4.78	19.69	4.54	23.11	3.83	14.25	4.38	0.29	19.02	2.58	14.63	2.36
	20.00	3.21	26.59	3.73	22.16	3.84	23.28	3.59	0.19	24.01	1.33	20.42	1.51

Tail Lenght (µm)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE
DMSO	5.00	5.13	6.88	6.98	7.47	7.78	8.07	6.63	0.79	7.47	0.34
	20.00	8.04	12.79	4.89	10.75	6.64	7.44	6.53	0.91	10.32	1.56
H_2O_2	**	65.73	64.59	52.65	64.08	44.17	62.42	54.18	6.27	63.70	0.65
A	1.00	8.07	8.05	7.07	7.86	5.54	8.09	6.89	0.74	8.00	0.07
	2.50	9.08	10.39	5.66	8.69	6.61	6.95	7.12	1.02	8.68	0.99
	5.00	7.40	8.85	5.59	9.68	6.46	8.74	6.48	0.52	9.09	0.30
	10.00	6.26	28.65	6.69	33.32	6.97	18.74	6.64	0.21	26.91	4.30
	20.00	6.90	40.63	5.78	30.20	5.97	34.90	6.22	0.35	35.24	3.02

Tail Moment (%)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE
DMSO	5.00	0.29	0.44	0.41	0.67	0.48	0.46	0.39	0.06	0.52	0.07
	20.00	0.58	1.01	0.30	0.78	0.41	0.60	0.43	0.08	0.80	0.12
H_2O_2	**	42.02	45.95	31.00	46.01	23.61	42.35	32.21	5.35	44.77	1.21
A	1.00	0.43	0.59	0.42	0.47	0.33	0.59	0.39	0.03	0.55	0.04
	2.50	0.58	0.85	0.35	0.61	0.43	0.46	0.45	0.07	0.64	0.12
	5.00	0.45	0.58	0.38	0.80	0.44	0.59	0.43	0.02	0.66	0.07
	10.00	0.53	6.60	0.55	9.67	0.42	3.62	0.50	0.04	6.63	1.75
	20.00	0.37	12.71	0.35	7.83	0.34	8.58	0.35	0.01	9.71	1.52

** H_2O_2 concentration at 200µM.

Table F6 – Alkaline comet assay with FPG data, of 3 independent experiments, for sample M.

								DNA in Tail (%)					
	Concentration	1st Assay		2nd Assay		3rd Assay		Mean		Mean		FPG sensitive	
Sample	(µl/ml)	without FPG	with FPG	without FPG	with FPG	without FPG	with FPG	without FPG	SE	with FPG	SE	sites	SE
DMSO	0.00	1.84	3.09	2.70	2.78	3.35	3.80	2.63	0.43	3.22	0.30	0.59	0.34
	5.00	1.75	3.64	2.36	2.67	3.00	5.97	2.37	0.36	4.09	0.98	1.72	0.77
	20.00	3.20	5.10	2.98	2.59	2.37	3.49	2.85	0.25	3.73	0.73	0.88	0.67
H_2O_2	**	51.79	66.77	45.91	63.68	53.52	65.98	50.41	2.30	65.48	0.93	15.07	1.54
M	1.00	1.98	4.76	3.45	5.31	3.63	5.55	3.02	0.52	5.21	0.24	2.19	0.30
	2.50	2.48	17.60	4.67	8.55	5.86	9.21	4.34	0.99	11.78	2.91	7.45	3.84
	5.00	11.35	32.14	4.28	15.15	4.52	10.57	6.72	2.32	19.29	6.56	12.57	4.34

						Tail Lenght (µm)					
	Concentration	1st Assay		2nd Assay		3rd Assay		Mean		Mean	
Sample	(µl/ml)	without FPG	with FPG	without FPG	with FPG	without FPG	with FPG	without FPG	SE	with FPG	SE
DMSO	0.00	3.13	4.68	4.59	5.33	6.12	4.71	4.61	0.86	4.91	0.21
	5.00	2.47	5.27	4.47	3.54	8.27	8.79	5.07	1.70	5.87	1.54
	20.00	5.54	8.06	4.35	3.82	4.78	5.39	4.89	0.35	5.76	1.24
H_2O_2	**	44.41	68.14	39.21	57.77	47.99	54.51	43.87	2.55	60.14	4.11
M	1.00	3.74	6.94	4.74	7.34	7.86	8.81	5.45	1.24	7.70	0.57
	2.50	3.78	17.92	5.45	10.80	12.26	14.56	7.16	2.59	14.43	2.05
	5.00	12.58	37.00	5.33	17.82	7.13	12.93	8.35	2.18	22.58	7.35

						Tail Moment (%)					
	Concentration	1st Assay		2nd Assay		3rd Assay		Mean		Mean	
Sample	(µl/ml)	without FPG	with FPG	without FPG	with FPG	without FPG	with FPG	without FPG	SE	with FPG	SE
DMSO	0.00	0.12	0.20	0.24	0.23	0.34	0.27	0.23	0.06	0.23	0.02
	5.00	0.11	0.29	0.18	0.15	0.36	0.64	0.22	0.08	0.36	0.15
	20.00	0.30	0.53	0.21	0.18	0.20	0.27	0.24	0.03	0.33	0.11
H_2O_2	**	25.92	49.15	20.49	38.97	28.51	37.55	24.98	2.36	41.89	3.65
M	1.00	0.15	0.47	0.26	0.54	0.50	0.59	0.30	0.10	0.53	0.04
	2.50	0.19	3.64	0.40	1.05	0.98	1.57	0.52	0.24	2.08	0.79
	5.00	3.53	14.47	0.40	3.18	0.46	2.02	1.46	1.03	6.56	3.97

** H_2O_2 concentration at 200µM.

Table F7 – Alkaline comet assay with FPG data, of 3 independent experiments, for sample Mf.

DNA in Tail (%)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE	FPG sensitive sites	SE
DMSO	5.00	3.12	4.40	3.69	5.51	4.17	4.29	3.66	0.30	4.73	0.39	1.07	0.50
	20.00	4.29	6.06	3.68	5.67	3.31	4.85	3.76	0.29	5.53	0.36	1.77	0.13
H_2O_2	**	59.00	66.15	53.97	65.65	48.54	63.96	53.84	3.02	65.25	0.66	11.42	2.39
Mf	1.00	5.24	3.83	3.60	5.08	3.28	5.01	4.04	0.61	4.64	0.41	0.60	1.01
	2.50	4.38	3.80	3.59	5.49	3.86	4.62	3.94	0.23	4.64	0.49	0.69	0.72
	5.00	2.54	4.92	2.58	4.75	5.26	4.21	3.46	0.90	4.63	0.21	1.17	1.11
	10.00	3.48	7.35	3.47	7.96	3.86	5.61	3.60	0.13	6.97	0.70	3.37	0.83
	20.00	3.81	7.74	3.96	7.31	4.05	6.53	3.94	0.07	7.20	0.35	3.26	0.42

Tail Lenght (µm)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE
DMSO	5.00	5.13	6.88	6.98	7.47	7.78	8.07	6.63	0.79	7.47	0.34
	20.00	8.04	12.79	4.89	10.75	6.64	7.44	6.53	0.91	10.32	1.56
H_2O_2	**	65.73	64.59	52.65	64.08	44.17	62.42	54.18	6.27	63.70	0.65
Mf	1.00	11.83	5.80	6.18	6.87	5.00	7.31	7.67	2.11	6.66	0.45
	2.50	8.73	6.04	4.82	8.19	5.16	7.26	6.24	1.25	7.16	0.62
	5.00	4.18	8.83	4.22	7.46	10.53	5.45	6.31	2.11	7.25	0.98
	10.00	5.60	10.85	6.22	12.89	6.35	8.38	6.06	0.23	10.71	1.31
	20.00	4.66	10.70	5.89	11.83	5.22	11.47	5.26	0.36	11.33	0.33

Tail Moment (%)

Sample	Concentration (µl/ml)	1st Assay without FPG	1st Assay with FPG	2nd Assay without FPG	2nd Assay with FPG	3rd Assay without FPG	3rd Assay with FPG	Mean without FPG	SE	Mean with FPG	SE
DMSO	5.00	0.29	0.44	0.41	0.67	0.48	0.46	0.39	0.06	0.52	0.07
	20.00	0.58	1.01	0.30	0.78	0.41	0.60	0.43	0.08	0.80	0.12
H_2O_2	**	42.02	45.95	31.00	46.01	23.61	42.35	32.21	5.35	44.77	1.21
Mf	1.00	0.74	0.37	0.32	0.50	0.29	0.53	0.45	0.15	0.47	0.05
	2.50	0.54	0.35	0.29	0.58	0.34	0.46	0.39	0.08	0.46	0.07
	5.00	0.19	0.58	0.20	0.52	0.69	0.38	0.36	0.17	0.49	0.06
	10.00	0.34	1.01	0.37	1.41	0.45	0.65	0.39	0.03	1.02	0.22
	20.00	0.30	1.15	0.36	1.28	0.35	1.02	0.34	0.02	1.15	0.07

** H_2O_2 concentration at 200µM.

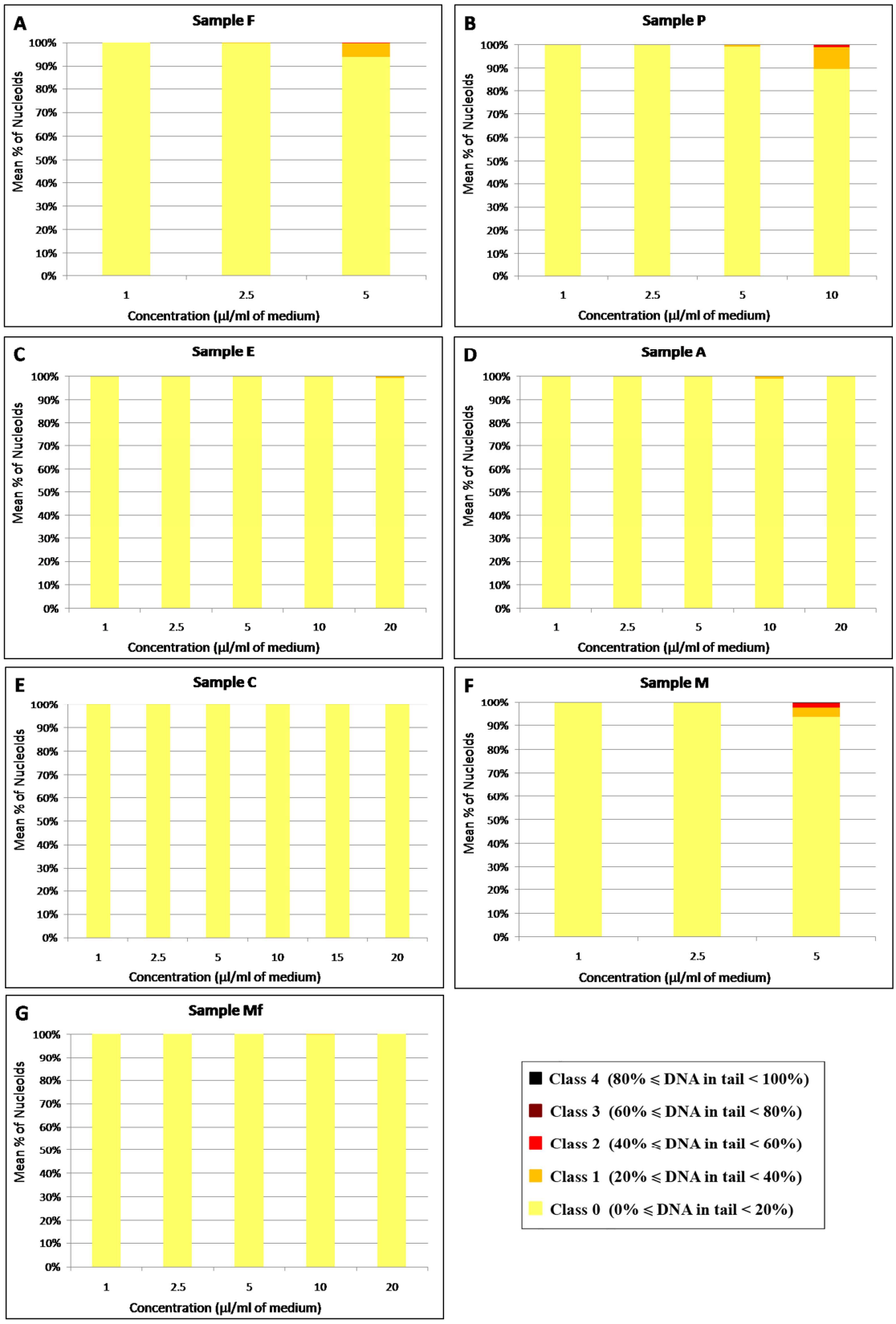

Figure F1 – Distribution of the mean percentage of observed nucleoids per classes. Classes were obtained from 3 independent experiments, according to the percentage of DNA in tail obtained for each sample, without FPG treatment. **A** – Sample F; **B** – Sample P; **C** – Sample E; **D** – Sample A; **E** – Sample C; **F –** Sample M; **G** – Sample Mf.

ANNEX G – Experimental data obtained in the micronucleus assay following a 24h exposure period to sediment extracts.

Table G1 – Experimental data obtained in the micronucleus assay, following 24h exposure to samples F, C and M.

	Concentration	1st Culture			2nd Culture			Data Per Treatment							
Sample	(µl/ml)	Nº MN	Nº NBUDs	Nº NPBs	Nº MN	Nº NBUDs	Nº NPBs	Total MN	SD	Total NBUDs	SD	Total NPBs	SD	CBPI	RI
DMSO	5.00	18	4	5	22	4	3	20	2.83	4	0.00	4	1.41	1.78	N/A
Mytomycin	**	79	5	2	72	3	2	76	4.95	4	1.41	2	0.00	1.35	45.11
F	1.00	25	0	2	21	3	4	23	2.83	2	2.12	3	1.41	1.62	79.87
	2.50	36	0	2	26	4	7	31	7.07	2	2.83	5	3.54	1.47	60.77
	5.00	29	3	6	35	9	6	32	4.24	6	4.24	6	0.00	1.27	34.66
C	1.00	15	0	3	15	2	7	15	0.00	1	1.41	5	2.83	1.71	90.27
	2.50	17	3	3	16	5	7	17	0.71	4	1.41	5	2.83	1.68	86.57
	5.00	18	3	0	23	3	1	21	3.54	3	0.00	1	0.71	1.53	67.57
M	1.00	16	2	3	7	2	7	12	6.36	2	0.00	5	2.83	1.67	85.30
	2.50	21	4	5	37	5	3	29	11.31	5	0.71	4	1.41	1.57	72.97
	5.00	40	4	9	29	6	12	35	7.78	5	1.41	11	2.12	1.39	49.81

** Mytomycin C at concentration 0.3µg/ml.