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Environmental and human risk assessment of landfill leachate: An integrated approach with the use of cytotoxic and genotoxic stress indices in mussel and human cells

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HIGHLIGHTS

- Landfill leachate poses a threat for aquatic biota and humans.
- Leachate induces cytotoxic and oxidative effects on mussel hemocytes.
- Increased levels of DNA damage were observed both *in vivo* and *in vitro* in hemocytes.
- Leachate low doses enhance MN formation in human lymphocyte cultures.
- Potential leachate aneugenic activity was detected in human lymphocytes.

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ABSTRACT

The present study investigates leachate hazardous effects on marine biota and human cells, with the use of a battery of assays, both under *in vivo* and *in vitro* conditions. According to the results, mussels exposed for 4 days to 0.01 and 0.1% (v/v) of leachate showed increased levels of DNA damage and micronuclei (MN) frequencies in their hemocytes. Similarly, enhanced levels of DNA damage were also observed in hemocytes treated *in vitro* with relevant concentrations of leachate, followed by a significant enhancement of both superoxide anions ($^{\bullet}O_2^{-}$) and lipid peroxidation products (malondialdehyde/MDA). On the other hand, human lymphocyte cultures treated with such a low concentrations of leachate (0.1, 0.2 and 1%, v/v), showed increased frequencies of MN formation and large MN size ratio, as well as decreased cell proliferation, as indicated by the use of the cytokinesis block micronucleus (CBMN) assay and Cytokinesis Block Proliferation Index (CBPI) respectively. These findings showed the clear-cut genotoxic and cytotoxic effects of leachate on both cellular types, as well as its potential aneugenic activity in human lymphocytes.

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1. Introduction

During the last decades, ecotoxicological and toxicological methodologies have become a priority worldwide, in order to assess the human and environmental risk of pollutants and mixtures of contaminants [1]. Among them, landfill leachate, commonly generated by the precipitation and penetration of water into the mass of residues in disposal landfill areas [2], is considered an environmental matter of concern [3]. This type of liquid effluent consists of a huge number of pollutants, such as dissolved organic matter, inorganic salts, heavy metals and xenobiotic organic compounds [4] which could be toxic and carcinogenic [5] and able to induce potential risk for biota and humans.

The entrance of leachate into ground- and surface waters could render them unusable for drinking and other domestic purposes [6]. On the other hand, bearing in mind that leachate chemical compounds can easily assimilate by aquatic organisms, pass through the food chain and bioaccumulate over long-term exposure [7], the human population is increasingly under threat, after consumption of contaminated water and/or food [8,9].

Landfill leachate ability to cause deleterious and genotoxic effects upon organisms of various trophic levels, including crustaceans [10–12], fish species [13,14], bacteria [15], plants [16–19], mice [20] and human [6,21–23] is well known, but only recently reported its ability to enhance oxidative and genotoxic effects on marine bivalve molluscs, such as *Mytilus galloprovincialis* [24].

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Those bivalves accumulate large amount of contaminants in their tissues, thus allowing their use as biological models for assessing water and sediment quality [25]. Since mussels are widely cultivated and consumed by humans, their use as target organisms for monitoring biological responses, commonly called stress indices, discloses the environmental impact of contaminants, such as leachate, not usually and/or hardly analyzed in the real environment [24,26], as well as their potential human risk [27].

Regarding that leachate environmental and human risk depends on its physico-chemical characteristics, the estimation of stress-indices in organisms from different trophic levels could assess the adverse effects of leachate chemical compounds on human and ecosystem health. According to the latter, the present study investigates leachate composition and its toxic effects on mussel hemocytes and human lymphocytes, both involved in the biological/immune response against xenobiotics. In specific, a battery of cytotoxic, oxidative and genotoxic stress indices were determined for a first time in mussel hemocytes, after *in vivo* and *in vitro* exposure to different concentrations of leachate, since this cellular type is widely used for investigating cellular responses, thus indicating the immunosurveillance and the concomitant health status of the organism [28,29]. On the other hand, leachate-mediated cytotoxic and genotoxic effects were investigated in human lymphocytes by the use of the cytokinesis block micronucleus (CBMN) assay, widely performed for assessing the risk of different types of chemical substances and their ability to cause genetic damage and carcinogenetic processes in humans [30,31].

2. Experimental

2.1. Chemicals and reagent

All reagents and solvents used were of the highest analytical grade and purity (see SM 2.1).

2.2. Leachate collection and handling

Both *in vivo* and *in vitro* studies were conducted with the use of well-characterized leachate [24] (see also SM Table 1), collected every 10 days within November 2011 from the landfill (active) site of Aigeira (Eastern Aigialeia, Peloponissos, Greece). In specific, equal volumes of all samples (at least 5 L) were pooled, filtered through sterilized filter membranes (diameter 0.2 μm) and frozen at −28 °C in the dark until used.

2.3. Mussel collection and handling

2.3.1. Ethics statement

The Mediterranean mussel *M. galloprovincialis* (Lmk. 1819) is common and not endangered invertebrate species. Since no permits were required for their use in both *in vitro* and *in vivo* studies, the experimental procedure (in terms of acclimation period, mussel handling and exposure procedure) was appropriately carried out in order to minimize animal suffering. In brief, mussels (4–5 cm long) were collected from a mussel-farm located to the north side of Korinthiakos Gulf (Gulf of Kontinova, Galaxidi, Greece) in March 2012 (no interference of massive spawning is expected), transferred and acclimated under laboratory conditions for 7 days in static tanks, containing aerated (dissolved oxygen 7–8 mg L^{-1} at 15 °C and 35‰ salinity), recirculated UV-sterilized and filtered artificial sea water (ASW). Mussels maintained without feeding during the acclimation period and then fed daily (approximately 30 mg of dry-microencapsules, Myspat, Inve Aquaculture^{NV}, Belgium/mussel).

2.4. In vivo exposure of mussels to sublethal concentrations of leachate

After the acclimation period, four groups of mussels (30 mussels/tank) were placed in static glass-tanks (30 L) and exposed for 4 days to sublethal concentrations (0.01 and 0.1%, v/v) of leachate, as recently mentioned [24]. Seawater was changed every day and new quantities of leachate and food were added in each tank. The aforementioned experimental procedure was repeated twice ($N = 2$).

2.4.1. MN assay

After the end of the exposure period, live mussels were removed and hemolymph was withdrawn from the posterior adductor muscle of 10 individuals of each group (control and leachate-exposed mussels), using a sterile 1 mL syringe with an 18 G1/2 in. needle, containing equal volume of Alseve buffer (ALS buffer; 20.8 g L^{-1} glucose, 8 g L^{-1} sodium citrate, 3.36 g L^{-1} EDTA and 22.5 g L^{-1} NaCl, pH 7 and 1000 mOsm L^{-1}). Thereafter, 40 μL of hemolymph suspension from each individual were spread on slides, in duplicate, transferred to a lightproof humidity chamber in order to attach, stained with Giemsa and finally scanned under an optical microscope (Zeiss microscope, 100× magnification) for the presence of micronuclei (MN) formation, according to the procedure and criteria proposed by UNEP/RAMOGE [25].

The hemolymph collected from the rest of 20 individuals per group of mussels was pooled, further divided in 3 subgroups (which mean 3 replicates/group of mussels and 6 replicates for the whole experimental design) and small portions were finally used for conducting Comet assay method (estimation of DNA damage).

2.5. In vitro treatment of mussel hemocytes with leachate

Hemolymph of laboratory acclimated mussels was withdrawn from the posterior adductor muscle of 10 individuals, filtered through sterile gauze and pooled in Falcon tubes at 4 °C. Hemolymph serum, used for hemocytes suspension, was obtained by centrifugation of whole hemolymph at 150 × g for 10 min and sterilization through a 0.22 μm-pore filter. Hemocyte suspensions/monolayers (at least 10^6 cells mL^{-1}) were incubated at 18 °C with different concentrations of leachate (0, 0.01, 0.1, 1, 10, 20, 40 and 80%, v/v, from stock solutions diluted in hemolymph serum) for 1 h. All experiments were performed in quadruplicate. Cell viability, tested with Eosin exclusion test (see SM 2.5) after cell dissociation and re-suspension in sterilized and filtered hemolymph, was about 95%.

2.5.1. Lysosomal membrane stability (neutral red retention assay)

Lysosomal membrane stability was evaluated with the use of neutral red retention time (NRRT) assay [32], based on the procedure and criteria proposed by UNEP/RAMOGE [25]. In brief, 40 μL of hemocytes suspension were spread on slides, in triplicate, transferred to a lightproof humidity chamber and allowed to attach. All slides containing cell monolayer were incubated with 40 μL of the cationic dye neutral red probe (40 μg mL^{-1}) for 15 min in dark, and thereafter were examined systematically under a light microscope every 15 min (at least 200 granular hemocytes examined in each slide). The time period between the NR probe application and the appearance of the first evidence of dye loss from the lysosomes to the cytosol or of other lysosomal abnormalities (Fig. 1) in at least 50% of the examined cells represented the NRR time.

2.5.2. Superoxide anion ($^{\bullet}O_2^{-}$) detection and lipid peroxidation (in terms of MDA equivalents) content in leachate-treated hemocytes

Superoxide anions ($^{\bullet}O_2^{-}$) were detected intracellularly by a modification of a previously described method [33], with the use of

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