Municipal leachates health risks: chemical and cytotoxicity assessment from regulated and unregulated municipal dumpsites in Lebanon

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Abstract

The proper management of municipal waste is critical for resource recovery, sustainability and health. Lebanon main approach for managing its municipal waste consisted of landfill disposal with minimal recycling capacity. This approach contributed to exceeding the holding capacity of existing landfills leading eventually to their closures. The closure of a major landfill (Naameh landfill) servicing Beirut and Mount Lebanon areas led to municipal wastes piling in the streets and forests for more than a year in 2016. The main problem identified in the municipal wastes consisted of untreated leachates (from regulated and unregulated dumpsites) going straight into the Mediterranean Sea. Therefore leachate samples were collected and subjected to chemical characterization followed by biological assessment. The chemical characterization and profiling of the Lebanese leachates were compared to results reported in Lebanon, Europe and United States as well as to the toxicity reference values (TRV). The biological assessment was conducted *in vitro* using human derived immortalized cell cultures. This strategy revealed significant alarming cellular organelles and DNA damages using *in vitro* cytotoxicity assays (MTS and comet assay). The significant damages observed at the cellular level prompted further animal model investigations using BALB/c mice. The animal data pointed to significant upregulation of liver activity enzymes coupled with significant damage expression in liver spleen and bone marrow

DNA. The presented research clearly indicated that there is an urgent need for development of national waste strategies for proper treatment and disposal of municipal waste leachates in Lebanon.

Keywords: Municipal waste, Leachates, toxicity, chemical characterization, in vitro assays, health impacts

1 – Introduction

Municipal waste disposal into landfills is the primary option adopted by numerous countries worldwide (Baderna et al., 2011) (Mukherjee et al., 2015) (Gong et al., 2014). This disposal method poses numerous challenges due to the unplanned development of landfill areas lacking adequate engineering controls or even improper oversaturation of existing landfills. This leads to the potential formation of toxic gases and leachates that can escape containment and find their way to the soil and groundwater (Mukherjee et al., 2015).

Leachates consists of the liquid effluent generated from municipal waste consolidation into landfills. This liquid is a cocktail of numerous chemicals that are the result of water passing through the waste and saturating it with organic and inorganic matter. The produced leachate poses significant disposal challenges for landfill operators worldwide due to its potential to contaminate soils, surface water, aquifers and sea water.

Leachate composition is affected by numerous factors as outlined in Johansen and Carlson 1976 (Johansen and Carlson, 1976). Briefly these factors include landfill age, the geological conditions present in addition to local weather affecting the hydrogeological conditions in the landfills. Other important parameters to be considered within the landfill includes interaction of various chemicals,

the internal temperature and pH. Landfill consolidated breakdown can occur under aerobic and anaerobic conditions. This breakdown contributes to stabilizing the organic component leading to lower leachates organic and inorganic concentrations (Jędrczak A. . 1994).

Landfill operators worldwide tend to compact the waste into landfills. This practice creates anaerobic conditions which results in methane gas formation. The formation of methane is observed in old landfills and is usually the result of acidogenic in young landfills followed by methanogenic reactions in old landfills. It is also important to note that the composition of the leachates together with climate and technology used in compacting the waste can play an important role in altering the leachates profile (Slomczyñska and Slomczyñski, 2004).

Many studies were conducted on landfill sites worldwide with a focus on mutagenicity (Deguchi et al., 2007) and toxicity mainly in plants (Bhat et al., 2016). Across northern Spain for example, four municipal solid waste landfill sites have been monitored for eleven perfluoroalkyl carboxylates (PFCAs) and five perfluoroalkyl sulfonates (PFSAs). PFSAs importance stems from their C-F bonds making them highly stable in long alkyl chains (Prevedouros et al., 2006). This property led to their extensive production and various applications including coating materials, water repellent surfactants and fire retardant. (Busch et al., 2010) (Dauchy et al., 2012) (Yan et al., 2015). PFSAs high stability makes them non-biodegradable, persistent and extremely difficult to remediate using non-conventional methodologies (Quiñones and Snyder, 2009) such as membrane bioreactors (Fotakis and Timbrell) with limited remediation success (Fuertes et al., 2017).

The cytotoxicity and DNA damage induction in four simulated landfill soil leachates from Nigeria and India were also evaluated. Theses assessments were conducted using the MTT cell proliferation assay for cell number determination assay and alkaline comet assay for the DNA damage assessment (Swati et al., 2017). Heavy metals (Cadmium, Iron and Zinc) (Ratzinger et

al.), Polycyclic Aromatic hydrocarbons (PAH), polycyclic chlorinated biphenyls (PCBs) and organic chemicals detected in samples were higher than allowable exposure limits. The researchers also reported significant cytotoxic and DNA damage induction in exposed cells leading to significant morphological alterations and apoptosis. All these results clearly indicated the significant health risks posed by leachates exposure (Alimba et al., 2016). The assessment of leachates from India also showed a similar pattern with reports of high level of organics (158 times allowable limits) and lower heavy metal content. The paper highlighted the significant health risks posed by low concentrations of PAH especially when these PAH interact synergistically to cause cytotoxic and genotoxic damages (Ghosh et al., 2015).

Leachates from landfills in Turkey were also assessed. Researchers reported varying pH (4-8), temperatures (2.8 and 24.5 ºC) and organic compounds (33 in all) such as phthalates and naphthalene and alkanes (Banar et al., 2006).

Lebanon leachates were previously analyzed and reported in a 2002 study by El-Fadel et al., (El-Fadel et al., 2002). The leachates were analyzed for a number of parameters such as pH, COD, Total organic Carbon (TOC), Total Dissolved Solid (TDS), chlorides, sulfates, orthophosphates, nitrates, ammonia nitrogen, hardness, and heavy metals.

Recently in 2016, municipal waste has been an emerging concern in our Lebanon. The regulated dumping sites could no longer manage the tremendous quantity of municipal garbage generated and therefore numerous unregulated dumping sites were created throughout the country. This large number of unregulated waste disposal areas could be correlated to environmental pollution and human diseases. To minimize the impact of dumpsites on human health and the environment, a qualitative and quantitative research into leachate production, toxicity and potential management was needed. It is important to note that waste leachate was typically released to the environment

without any treatment, increasing the risk of environmental and human damages. Consequently, we must improve our knowledge of such matrices to be able to develop reliable treatment processes.

The main challenges for leachates management were mainly toxicological in nature (Kalka, 2012) (Ghosh et al., 2017) (Slomczyñska and Slomczyñski, 2004). The leachates chemical composition was anticipated to vary between different dumping sites depending on the nature of the waste as well as the climate. Establishing toxicity profile predictions for these leachates is challenging due to the unique geochemical nature of each landfill and the variation in soil layers and water table (Koshy et al., 2007).

The uncontrolled disposal of these leachates into the soil and waterways poses significant challenges due to the composition of these leachates mainly in the form or organic and inorganic contaminants (Nagarajan et al., 2012) (Raghab et al., 2013). Leachates also poses pressures on Biochemical and Chemical Oxygen Demand (BOD and COD), TOC, ammonium and sulfur compositions and heavy metals in soil and groundwater (Gajski et al., 2012). The mixture of compounds generated by landfill is complex and more than 200 compounds of hazardous nature have been previously identified in landfill leachates. These compounds ranges from aromatic to phenols, halogenated and other compounds as described in the literature (Öman and Junestedt, 2008) (Baun and Christensen, 2004) (Adar and Bilgili, 2015).

The various chemical compounds in municipal leachates can lead to significant damages in ecological systems, food chains and ultimately human population. These effects can range from toxicity to carcinogenicity as reported by many researchers (Mukherjee et al., 2015) (Moraes and Bertazzoli, 2005) (Gajski et al., 2012).

A map showing the municipal dumps leachates collection sites can be referred to in Figure 1.

Figure 1: Map of Lebanon indicating leachates sampling sites marked as ***** on the map.

The main motivation behind the research was to highlight the significant health and environmental risks posed by disposal of untreated municipal leachates into the natural environment. We anticipate that the findings will guide decision makers in the country to adopt better waste management policies and strategies. The novelty of this study is at two levels. Firstly, the research aims at establishing a linkage between the Lebanese leachates chemical compositions and the biological impacts observed at the cellular and animal levels (not previously reported in the literature). Secondly, we could not find any published data on the genotoxic impacts of Lebanese municipal leachates and this is a very important salient matter to address. The leachates genotoxic data clearly indicated the urgent need for the government to address the issue due to the long-term public health implications. This a very important matter because leachates exposure could potentially lead to future cancer onsets in the Lebanese population.

2. Materials and methods

This study evaluated the leachates sampled from a number of regulated (Tripoli and Zahle) and unregulated municipal dumpsites (Ghazeer, Mount Lebanon) throughout Lebanon (El-Fadel, Bou-Zeid et al. 2002). The five samples were collected from three sites as shown in Figure 1 and consisted of the following:

1. Zahle old waste leachate, untreated from weathering pond next to landfill (sample A);
2. Zahle new waste leachate, untreated straight from landfill (sample B);
3. Tripoli waste leachates straight from Landfill, untreated (sample C);
4. Tripoli waste leachates biologically treated (sample D); and
5. Ghazeer waste leachates, unregulated municipal dumpsite untreated (sample E).

It is important to note that the selected sites didn't have any biological treatment except for Tripoli landfill. The study consisted in characterization of chemical composition of landfill leachates. The analytical assessment was followed by *in vitro* (cellular model) and *in vivo* (mice model) to establish the biological impacts of leachates exposures. It is important to note that the fate of treated and untreated landfill leachates in Lebanon was ultimately the Mediterranean Sea. The untreated leachates originated predominantly from inland and sea front landfills trucked in using major highways prior to disposal into the sea. Sea disposal was through a 1.5 km pipeline in an area categorized as dead zone known as Ghadir. This area was used for disposal of industrial and other waste discharges over many years.

2.1. Study design

Leachates were collected in glass bottles on ice prior to transportation to the labs using the standard method for leachate examination (American Public Health et al., 1998). Samples were processed for chemical characterization, and cytotoxicity assessment. The samples used for cytotoxicity

assessment were centrifuged at high speed to remove any particles and then filter sterilized using a 0.2 micrometer filters. The samples were then diluted prior to conducting the cytotoxicity and animal toxicity assessments.

2.2. Analytical assessment

Bis(2-ethylhexyl) phthalate, pestanal analytical standard was purchased from Sigma-Aldrich. The metal standards (1000 mg/L in 0.5 M HNO_3) were purchased from Merck whereas anions were purchased from Ultra Scientific. All HPLC/GC grade solvents and other chemicals were purchased from Sigma-Aldrich or Fisher Scientific and used without further purification.

2.2.1. Preparation of waste leachates organic compounds

The organic compounds were extracted from 50 mL of leachates with dichloromethane (DCM) solvent (500 mL, 3 times) using liquid-liquid extraction method. After extraction, the DCM solution was dried using calcium sulfate, filtered then evaporated using rotary evaporator under a pressure of 500 mBar and 40 ˚C. The resulting solid was reconstituted in 1 mL of DCM prior to GC/MS analysis. The tested samples were filtered with Cronus Syringe Filter PTFE 25 mm 0.45 µm to remove any suspended particles.

2.2.2. GC/MS analysis

Samples from municipal leachates were subjected to GC/MS using a GC/MS (Hewlett Packard, HP6890 fitted with a fused silica HP5-MS 5% phenyl methyl siloxane column (30 m x 0.25 mm) i.d., film thickness (0.25) and coupled to MS detector. Helium was the bearer gas used with splitless infusion (1 µL infusion volume) at a rate of 1.2 mL/min. The temperature program was 2.0 min at 70 °C, from 70 °C to 130 °C at 8 °C/min and hold for 5 min, from 130 °C to 180 °C at

2 °C/min and hold for 10 min, from 180 °C to 220 °C at 15 °C/min and hold for 2 min, from 220 °C to 280 °C at 15 °C/min and hold for 45 min and after that from 280 °C to 300 °C at 15 °C/min and hold for 20 min. The assessment was conducted in scan mode to broaden the search process. Compounds were identified using the NIST11 and W9 libraries. The presence of di(2-ethylhexyl) phthalate (DEHP) in the five samples was confirmed by GC/MS using its commercial form as standard, Figure S1-S2.

2.2.3. Chemical characterization and statistical analysis

Leachates samples were vacuum sucked through a 0.45 μm pore cellulose filters, divided into two samples in polyethylene bottles. One sample was acidified with nitric acid to pH < 2 and stored at 4 ºC for metal analysis (triplicates) (Pb, Cd, Cr, Cu, Al, Mn, Fe, Zn, As, K, Na and Ni) using AAS Graphite Furnace technique ("Shimadzu" AAS-6300). The working standards were prepared by stock solution dilution (1 mg metal/mL in 2% HNO_3) with MilliQ water. The second sample was used for determination of a number of parameters such as pH, conductivity (Hach Model 44600 Conductivity/TDS Meter, resolution Conductivity 0.1 μS/cm), turbidity (Nephelometric method), titration of Ca, Mg, total hardness (0.01 M EDTA), spectrophotometry for NO_3^- (cadmium reduction), SO_4^{2-} (turbidimetry), PO_4^{3-} (ascorbic acid), TDS, FS (fixed solid) and VS (volatile solid) using LOI (loss on ignition). The analysis were conducted with certified prepared reagents (EPA standards) obtained from Hach Chemical Company, USA. All procedures adopted were in accordance with the standard method of water analysis and waste water (APHA, AWWA, WPCF (2012)). Chloride Cl^- was determined by ion chromatography (Ion Chromatograph, "Shimadzu" PIA-100). TC (total carbon), TOC using a TC and TOC Analyzer "Shimadzu" VCPN.

2.3. Cytotoxicity assessment

2.3.1. Human modified keratinocyte cells (HaCaT)

Human HaCaT keratinocytes (non-tumorigenic) were derived from normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line (Boukamp et al., 1988). The cells were selected as they represented the first barrier in the skin in contact with leachates.

2.3.2. Cell culture protocols

Cells were maintained at 37 °C in a 5% CO_2 incubator. The cell culture medium used consisted of DMEM (Dulbecco's Modified Essential Medium; Gibco) with 5% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (0.1 mg/mL; Sigma, USA). Upon confluence cells were subjected to trypsinization and seeded at a density of 5 x 10^3 cells/mL. Cells viability surpassed 95% as shown by Tryptan Blue (Sigma, USA) analysis. The 24h post exposure assessment was conducted by further incubating exposed cells in fresh media for 24 h prior to assessment.

2.3.3. In vitro cytotoxicity assays

2.3.3.1. MTS assay

Cell mitochondrial dehydrogenase activity was undertaken using the MTS assay kit from Promega as previously described (Khalil, 2015) (Khalil and Winder, 2008).

2.3.3.2. Comet assay

The assay investigated the single stand and double strand DNA damages. The methodology used has been extensively described (Khalil and Shebaby, 2017).

2.4. Cell experimentation statistical analysis

All data presented in this paper represented the mean and standard deviation of three independent experiments with at least three replicates. Statistical analysis were conducted by Microsoft Excel, and Graphpad. Statistical significance was reported for $p < 0.05$.

2.5. Mice model assessment

The BALB/c mice model was selected as the biological indicator for assessment. Briefly mice were kept in cages in groups of five and maintained under a 12 h. photoperiod (08:00–20:00) at an environmental temperature of 22 °C ± 2 °C. The mice were deprived of normal drinking water and were provided instead with undiluted/diluted leachates as their main water source. The mice were exposed to leachates samples coming from the dumpsites where leachate treatment (microbial or environmental attenuation) was undertaken. The mice drank the leachate water for a period of 4 weeks prior to being sacrificed and analyzed for organ damages. During the trial period mice were weighed daily. All experimental protocols were approved by the Animal Ethical Committee of the Lebanese American University, which complies with the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2010). Blood was also collected from every mouse within each group directly from the inferior vena cava using a 5 mL syringe containing Na_2EDTA (1 mg/mL), and transferred to 5 mL tubes for analysis.

The samples were kept on ice and then were centrifuged at 3000 rpm for 30 mins. The supernatants were collected and tested for the alkaline phosphatase (ALP), glutamic-oxaloacetic acid transaminase (GOT) and glutamic-pyruvic acid transaminase (GPT) enzymes using standard commercial Spinreact kits.

Mice were sacrificed at the end of the exposure period and liver, spleen and kidneys were harvested. The organs were weighed on a calibrated balance and the results were recorded. Bone marrow cells were collected from animal carcasses using the method described in (Swamydas and Lionakis, 2013). Euthanized mice were subjected to surgery to remove the lower extremities. The femur and tibia were cleaned from any muscles and sprayed with 70% ethanol. The bones were placed in a Petri dish containing ice-cold RPMI 1640 1X supplemented with 10% FBS and 1% Penicillin/streptomycin. Bones were washed in ice cold PBS and broken in a sterile environment. Using a 12 cc syringe filled with RPMI supplemented with 10% FBS and 2 mM EDTA, the bone marrow was flushed out before being analyzed using the comet assay methodology.

The organs were also sliced and subjected to digestion in trypsin overnight at 4 ºC in a shaker. Cells from each organ were harvested the next morning though pipetting and subjected to the comet assay for DNA damage determination.

3. Results

3.1. Analytical chemistry

Five samples A, B, C, D and E were analyzed using GC/MS without dilution nor filtration prior to extraction. This ensured that no molecules were removed by pre-treatment and most information associated with the sample was retained. The extract was then filtered with 0.45 µm filter to remove any suspended particles prior to GC/MS injection. GC/MS was conducted on waste leachate organic composition (for the five samples) and chromatograms obtained. All the compounds listed (Table 1) in this part were identified using the NIST11 library, and matches between the experimental mass spectrum and theoretical ones were over 90% for every compound.

Compounds that were not confidently identified by the software did not exceed 3% by area of any sample mixture and were excluded from Table 1 for clarity.

Table 1: List of the compounds identified by GC/MS in samples A, B, C, D, and E. The relative integrated area percentage for each identified compound is tabulated and indicates the relative abundance of each compound in the extracted mixture.

Peaks number	Area %	Compound
Sample A		
1	56.84	Oleamide
2	30.66	Di(2-ethylhexyl) phthalate (DEHP)
3	12.51	Hentriacontane
Sample B		
1	29.42	Cyclic octaatomic sulfur
2	21.43	Oleamide
3	2.68	Hexanedioic acid, bis (2-ethylhexyl)ester also known as DEHA
4	43.89	DEHP
Sample C		
1	100	DEHP
Sample D		
1	68.27	Tributylamine (TBA)
2	13.74	Oleamide
3	18.00	DEHP
Sample E		
1	5.69	Nicotine
2	6.55	Hexanedioic acid, bis (2-ethylhexyl) ester also known as DEHA
3	2.35	Pentacosane
4	78.80	DEHP
5	5.69	Eicosane

The list of organic compounds extracted from the samples is not comprehensive. The heterogeneity of the original samples may be hindering the extraction of persistent organic pollutants especially the ones possessing low concentration in the aqueous layer. Other persistent organic pollutants (POPs) including PAHs, polychlorinated dibenzofurans (PCDFs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzothiophenes (PCDTs), polybrominated diphenyl ethers

(PBDEs) and PFASs which were detected in reported leachates samples, were typically undergoing a different extraction and detection procedures to the one adopted in this study (Gallen et al., 2017) (Dudzinska M R, 2011). DEHP which was not detected in the blank sample (Figure S1-S2), was common compound in all samples.

Advanced chemical characterization was also performed on leachate samples of the five sites and several metals were detected including Pb, Cd, Cr, Cu, Al, Mn, Cu, Zn and As (Figure 2).

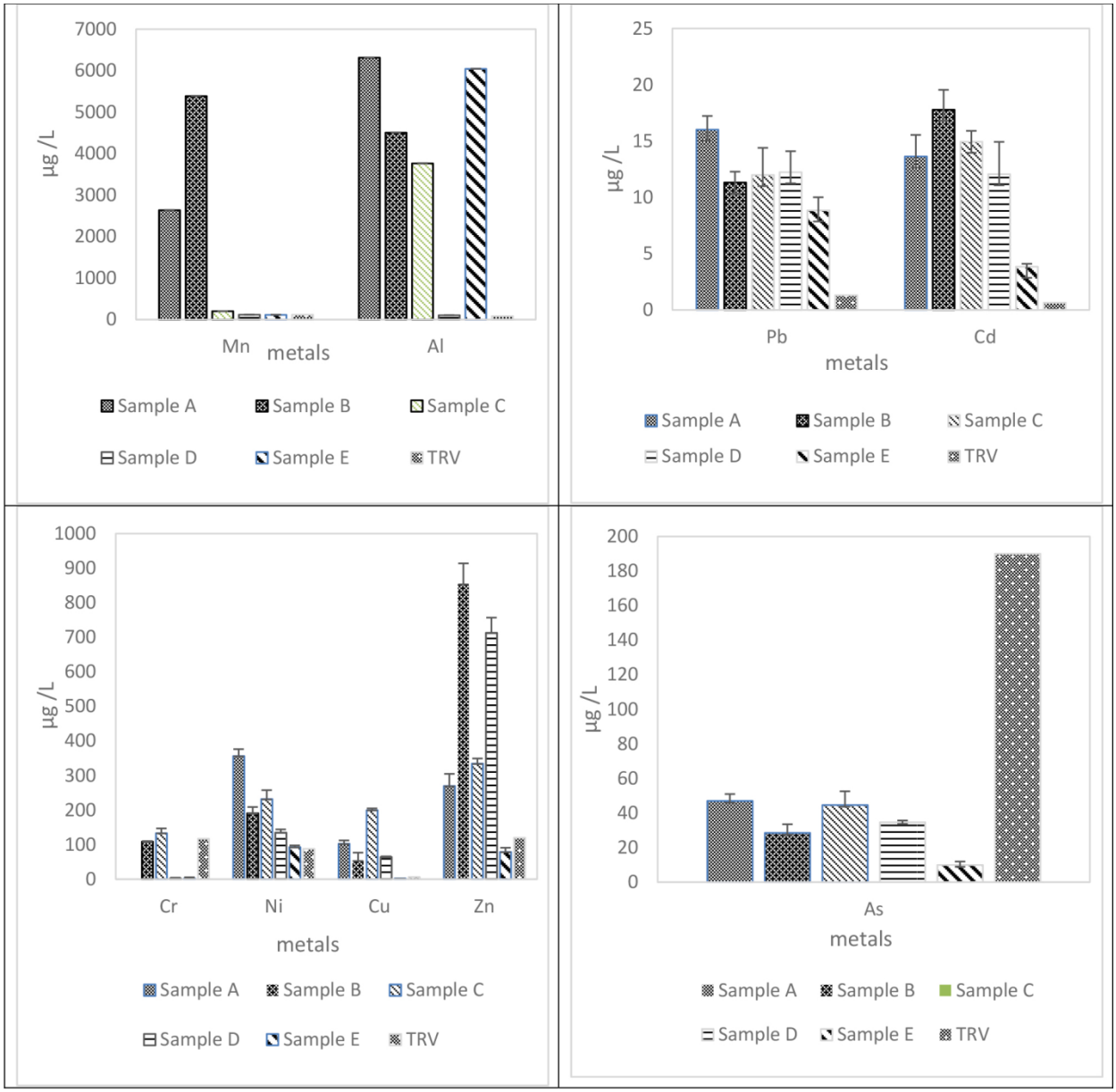

Figure 2: Variation of toxic metals among different samples with data related to TRV values.

Other important parameters were also evaluated such as K, Na, and Ni, NO_3^-, SO_4^{2-}, PO_4^{3-}, hardness, Cl^-, Ca, Mg, conductivity, turbidity, TOC, TC, IC, TDS, FS and VS (Table 2). The ranges of these parameters in all the five sites were calculated and compared to previously reported data in Lebanon (in year 2002), Norway and USA.

305 **Table 2:** Compositions of leachates from the five landfill sites compared to Lebanon (El-Fadel et al., 2002), Norway (Slomczyñska and
306 Slomczyñski, 2004) and USA (Johansen and Carlson, 1976)
307

Parameters	Units	Sample A	Sample B	Sample C	Sample D	Sample E	Range of samples A→E	TRV Unit: ppb = µg/L	Lebanon Unit: mg/L	Norway Unit: mg/L	USA Unit: mg/L
pH		7.85	7.44	8.12	6.34	5.81	5.81 - 8.12	N/A	1.5 - 9.5	5.9 - 7	5.4 - 6.4
Color true	**PtCo unit**	21800	26300	9875	600	7500	600 - 26300	N/A	N/A	N/A	N/A
Lead (Georgie J et al.)	**µg/L**	16.10	11.33	12.00	12.25	8.86	8.86 - 16.03	1.32	0 - 14.2	0.001 - 0.015	0.1 -1.40
Cadmium (Cd)	**µg/L**	13.65	17.80	14.95	12.08	3.86	3.86 - 17.80	0.66	0 - 1.16	0.0001 - 0.002	0.01 - 0.03
Chromium (Cr)	**µg/L**	-	10.88	133.33	22.30	24.50	2.23 - 133.33	117.32	0 - 22.5	0.002 - 0.17	0.05 - 1.05
Copper (Cu)	**µg/L**	102.50	53.85	199.80	64.28	2.47	2.47 - 199.80	6.54	0 - 9.9	0.008 - 0.085	0.18 - 1.30
Aluminum (Al)	**µg/L**	6316	4507	3767	107	6046	107 - 6316	87	0.5 -85.0	N/A	N/A
Manganese (Mn)	**µg/L**	2640	5389	205	120	117	117 - 5389	120	0.05 - 1400	N/A	N/A
Iron (Fe)	**µg/L**	9634	9543	8451	1741	4692	1741 - 9634	1000	0 - 42000	11.5 - 234	245 - 810
Zinc (Zn)	**µg/L**	270	854	334	713	79	79 - 854	120	0 - 1000	0.055 - 2.65	5.3 - 155.0
Arsenic (As)	**µg/L**	47.00	28.48	44.69	34.65	9.87	9.87 - 46.98	190	0 -70.2	N/A	N/A
Potassium (K)	**µg/L**	9162	8945	9805	8568	3704	3704 - 9805	53000	0.16 - 3370	21.3 - 219	N/A
Sodium (Na)	**µg/L**	9161	8945	9804	8567	3704	3704 - 9804	680000	0 - 8000	34.8 - 462	N/A
Nickel (Ni)	**µg/L**	356.50	191.00	231.80	135.00	92.70	92.68 - 356.50	87.71	0 -7.5	0.005 - 0.12	0.10 - 1.20

Parameters		Units	Sample A	Sample B	Sample C	Sample D	Sample E	Range of samples A→E	Lebanon Unit: mg/L	Norway Unit: mg/L
Nitrate - (NO_3^-)		mg/L	2	8	2	101	23	2 - 101	0 -9.8	N/A
Phosphate- (PO_4^{3-})		mg/L	41.00	50.25	32.65	8.00	48.10	8 00- 50.25	0.01 - 154	N/A
Sulfate- (SO_4^{2-})		mg/L	6	100	525	20	1525	6 - 1525	0 - 1850	10 - 100
Chlorides- (Cl^-)		mg/L	650	775	500	115	650	115 - 775	11375	68 - 680
Calcium- (Ca)		mg/L	62062	94094	12012	30030	230230	12012 - 230230	5 - 4080	99 - 400
Magnesium- (Aliahmadi et al.)		mg/L	10010	29136	7284	12140	18210	7284 - 29136	0 -115600	13 - 96
Water hardness	Total	mg/L	180000	60000	125000	650000	355000	60000 - 650000	N/A	N/A
	Calcium	mg/L	155000	30000	75000	575000	235000	30000 - 575000	($CaCO_3$) 0.1 -225000	N/A
	Magnesium	mg/L	25000	30000	50000	75000	120000	50000 - 120000	N/A	N/A
Conductivity		µs/cm	19260	23400	40300	1706	36000	1706 - 40300	480 -72500 (µs/cm)	N/A
Turbidity		NTU	320	310	96.25	36.24	1184	36.24 - 1184	40 - 500	N/A
TOC- (Total Organic Carbon)		mg/L	878	990	278	2	506	2 - 1184	335000	30 - 1700
TC- (Total Carbon)		mg/L	1415	1510	602	45	621	45 - 1510	N/A	N/A
IC- (Inorganic Carbon)		mg/L	537	500	304	43	98	43 - 537	N/A	N/A
TDS -(Total Dissolved Solid)		mg/L	26730	34700	12350	1100	17520	1100 - 34700	584 -55000	N/A
FS- (Fixed Solid)		mg/L	12690	16050	8030	600	10310	600 - 16050	N/A	N/A
VS- (Volatile Solid)		mg/L	14040	18650	3320	500	7210	500 - 18650	N/A	N/A

3.2. Cytotoxicity results

3.2.1. Leachates exposure

The viability of cells exposed to leachates was assessed using the MTS assay. Cells were treated with different filter sterilized dilution of raw leachates for 2 h. (immediately) and 24 h. from samples A, B, C, D and E and cytotoxicity assessment of leachates were evaluated using the MTS assay (Figure 3).

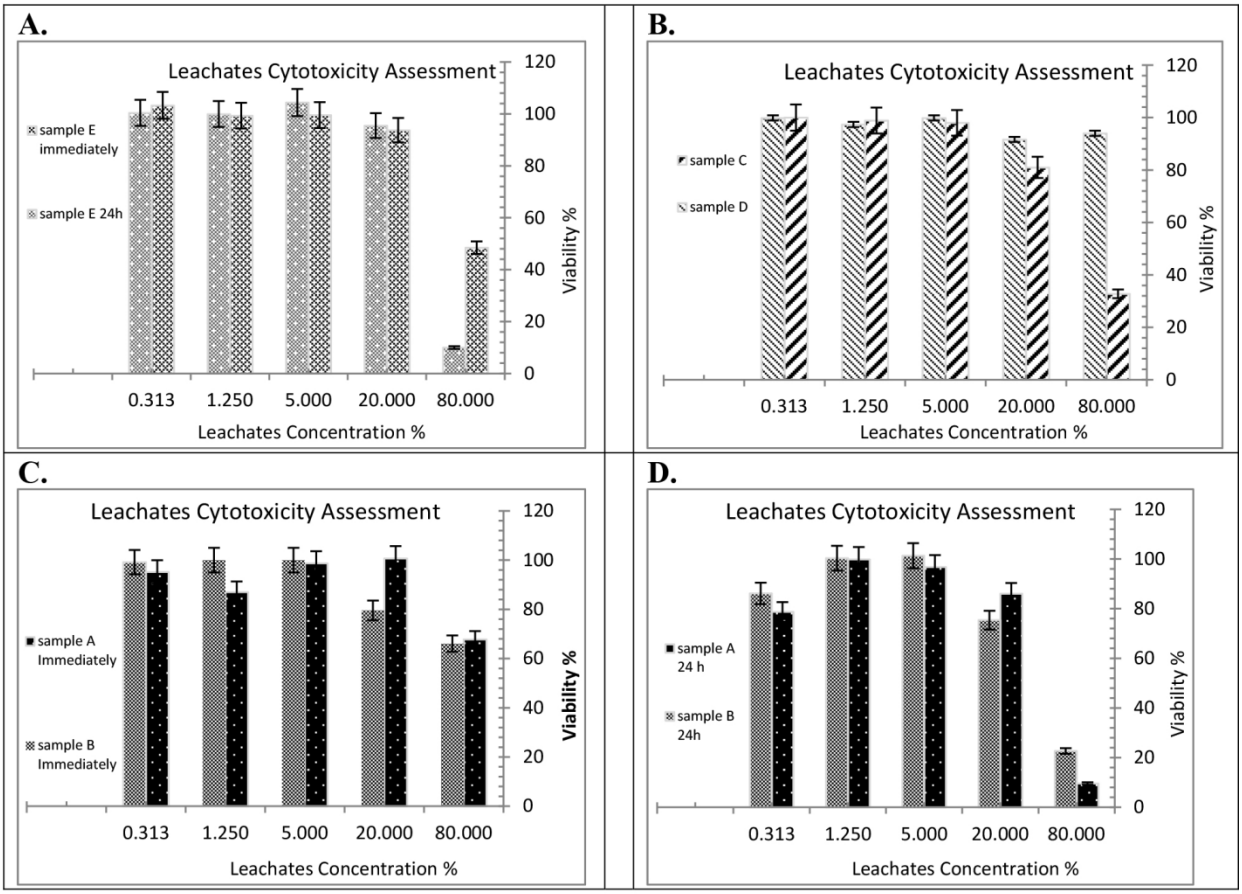

Figure 3: Leachate Cytotoxicity assessment from various locations throughout Lebanon.

The cytotoxicty results from the MTS assay and the expression of significant toxicity at 80% dilution immediately and 24 h. post exposure warranted the investigation of potential DNA damages triggered by leachates exposure. The aim of this approach was to determine whether a

similar profile to cytotoxicity analysis could be observed in DNA damage triggered by leachates exposure.

3.2.2. Leachates DNA damaging potential on cell cultures using comet assay

The DNA damage for the five samples was assessed using alkaline comet assay (Table 3). The DNA damage in cells exposed to leachates was shown using seven parameters such as head length, tail length, comet length, head DNA content, tail DNA content and tail moment.

Table 3: DNA damage analysis of HaCaT cells exposed to leachates. This was measured by the comet assay immediately (4h) post exposure with CASP (Comet assay Software package).

	Positive control	Negative control	Sample A	Sample B	Sample C	Sample D	Sample E
Head length	75.66 ± 9.68	115 ± 12.16	81 ± 20.13	119 ± 30.02	67 ± 3.05	81 ± 11.01	87 ± 10.06
Tail length	97.66 ± 12.11	4 ± 1	129 ± 11.01	135.6 ± 39.33	92.66 ± 4.33	69.66 ± 7.17	106.6 ± 5.54
Comet length	173.3 ± 18.02	119 ± 12.28	210 ± 24.84	254.6 ± 68.67	159.66 ± 6.74	150.66 ± 5.66	193.6 ± 12.70
Head DNA content	27.07 ± 5.76	99.63 ± 0.19	26.33 ± 5.32	43.67 ± 5.58	26.20 ± 1.03	45.44 ± 8.27	30.05 ± 4.67
Tail DNA content	72.92 ± 5.76	0.36 ± 0.19	73.66 ± 5.32	56.32 ± 5.58	73.79 ± 1.03	54.55 ± 8.27	69.94 ± 4.67
Tail moment	72.51 ± 13.95	0.01 ± 0.006	94.12 ± 5.23	73.21 ± 15.33	68.40 ± 3.48	39.16 ± 10.18	74.44 ± 5.13
Overall tail moment	45.56 ± 9.03	0.18 ± 0.08	60.27 ± 4.12	56.7 ± 10.01	44.56 ± 1.62	29.67 ± 3.90	59.20 ± 12.12

The tail moment Index (TMI) was calculated to reflect DNA damage in exposed HaCaT cells (Figure 4).

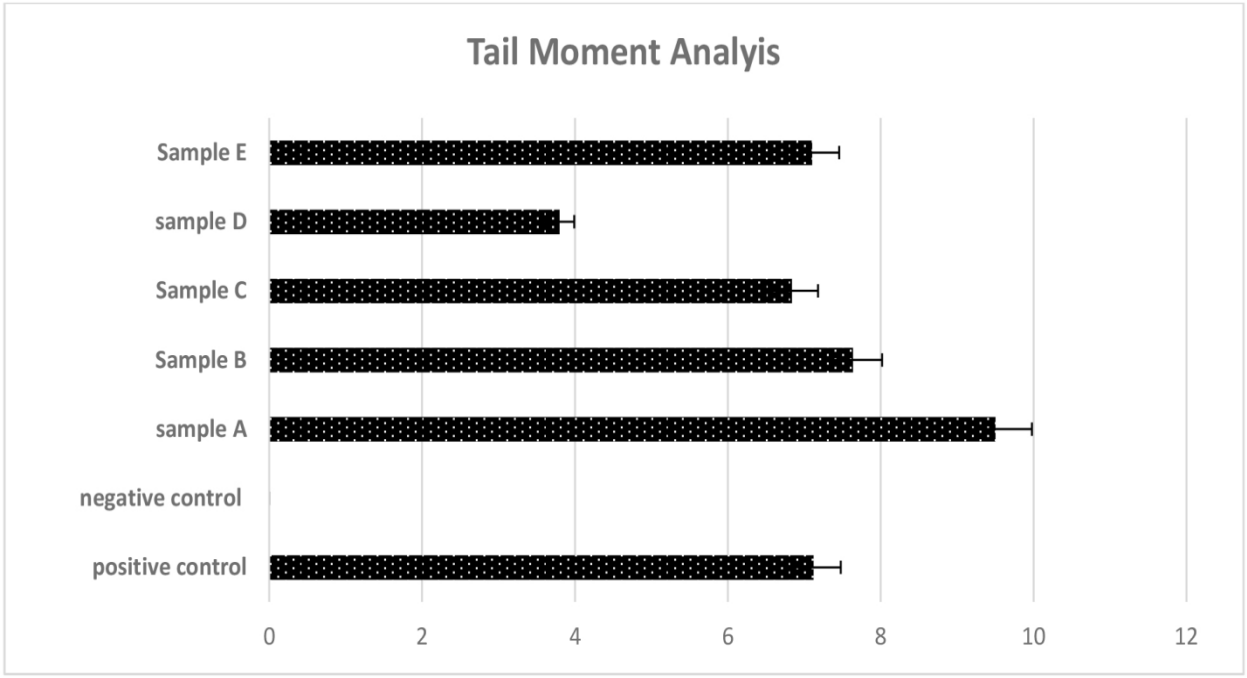

Figure 4: Tail moment analysis of comet assay data from leachate samples.

3.3. Leachates toxicity assessment using mice model

Mice were exposed to leachates as previously described in the material and method section. Average mouse overall weight in exposed and control mice was reported in Figure 5a. In controls the average weight was 19 grams, in sample A 23 grams, in sample B 21 grams, in sample C 19 grams, and in sample D 19 grams.

The organs of the mice were also harvested, and the weight of liver, kidney and spleen were also reported in Figure 5b.

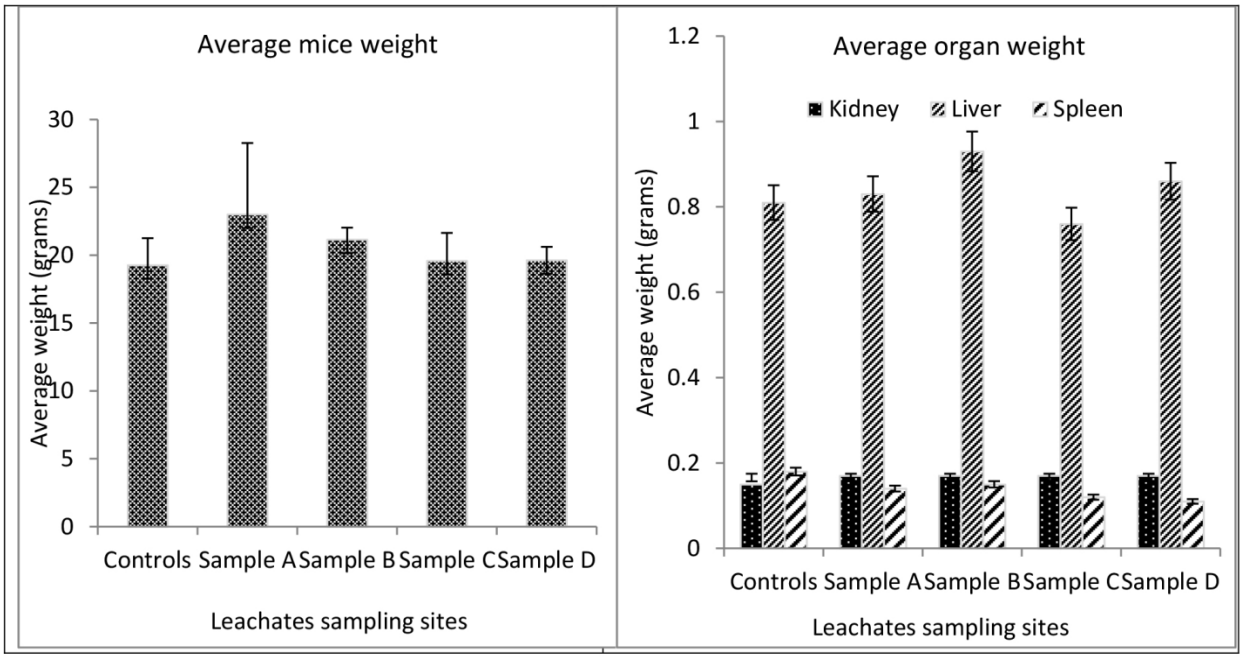

Figure 5: Mice overall and organ weights upon leachates exposure. The liver and blood of sacrificed mice was collected and subjected to several tests to establish enzymatic activities (Figure 6).

The ALP, GOT and GPT enzymes activities of the blood (ALP, GOT and GPT-100%) and the liver (ALP, GOT and GPT-L) of exposed and control mice was reported in Figure 6. We recorded significant increases in GOT levels upon leachates exposures in all samples.

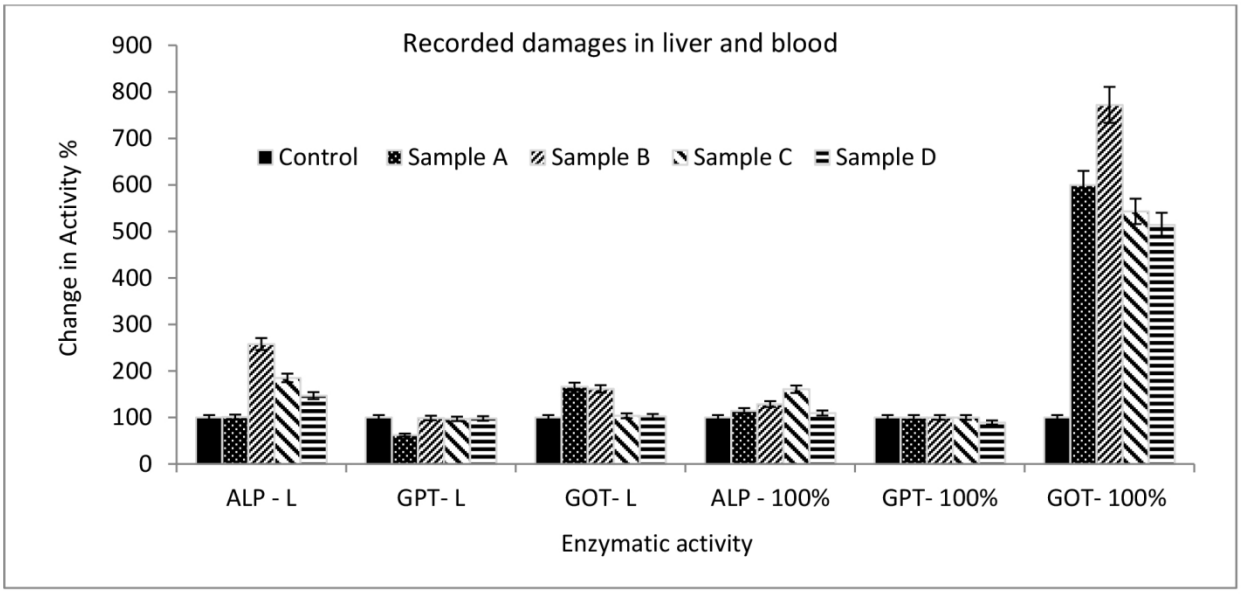

Figure 6: Enzymatic activity in blood of treated and untreated mice

The DNA damaging potential of leachates drinking was assessed by harvesting the DNA from isolated bone marrow, liver and kidney cells from exposed and control animals. The calculated TM index (details in section 3.2.2) was reported in Figure 7.

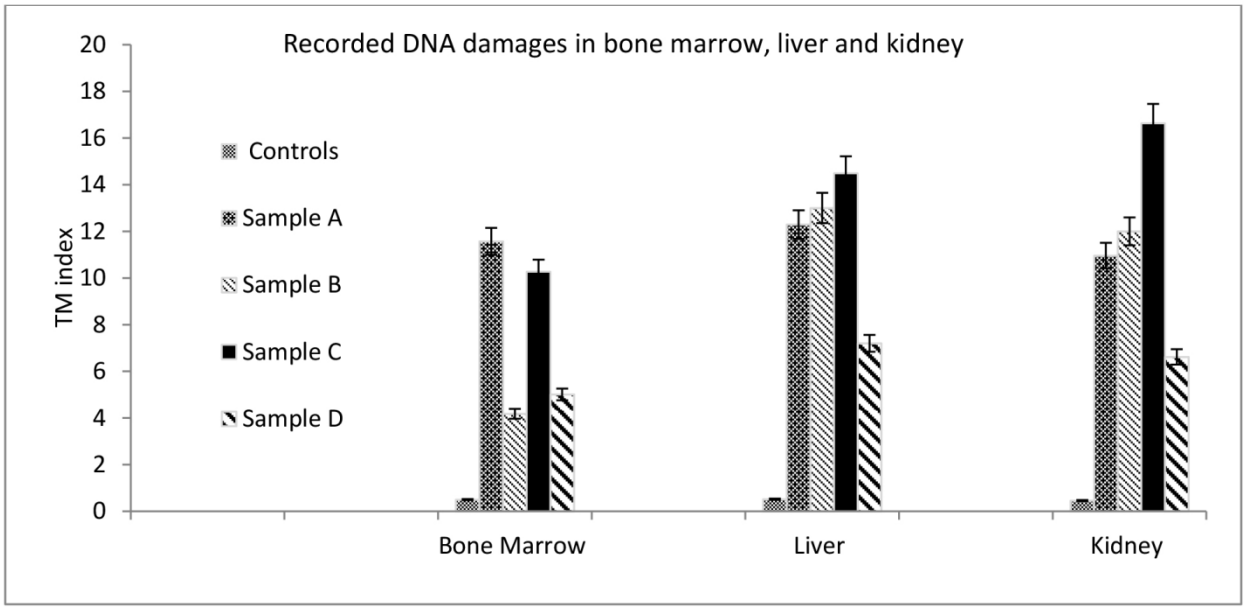

Figure 7: Tail moment index indicative of DNA damages in treated and untreated mice

4- Discussion

4.1. Leachates chemical characterization

The chemical characterization of the leachate samples led to identification of numerous compounds that were absent from the blank chromatogram as presented in Table 1, Figure S2. This blank was performed following the same extraction procedures but excluding the leachate sample presence, Figure S2. Leachate samples from different dumpsites had common chemicals. Among the identified chemicals oleamide, identified in samples A, B and D is reported as a sleep-inducing amidated lipid found in the cerebrospinal fluid of sleep-deprived cats (Cravatt et al., 1995) (Gobbi et al., 1999) in addition to its use as a slip agent in polyethylene filaments (Garrido-

López et al., 2006). Another common compounds were n-alkanes (hentriacontane and eicosane) mainly identified in samples A and E. Eicosane is commonly used in cosmetic, lubricants and plasticizers (Larranaga, 2016). Solid n-eicosane (paraffin waxes) are also used as feeds for cracking gasoline in addition to its use in oxidation, and chlorination cracking reactions (Schmidt, 2016). Eicosane was also reported as a principal component of diesel exhaust nanoparticles (Kanno et al., 2008). Other identified chemical compounds such as Hexanedioic acid, bis(2-ethylhexyl)ester or DEHA (in samples B and E) is used in the form of DEHP in medical products, children's products, and plastic cling wrap for food storage (Rodgers et al., 2014) (Testai et al., 2016), DEHA triggers slight irritation (in rabbit) and shows mild acute toxicity (Van Vliet et al., 2011) and is classified Category 3 carcinogen by the International Agency of Research on Cancer (Cancer) (Van Vliet et al., 2011) (Cancer, 2000). Tributylamine (TBA), identified in sample D, is used as an inhibitor in hydraulic fluids; and chemical intermediate (Larranaga, 2016). DEHP identified in samples A, B, C, D and E, is a hazardous plastic additive hazardous to human health and reproductive system(Lithner et al., 2009) (Castillo and Barceló, 2001). DEHP is also present in plastic products (wall coverings, furniture upholstery, garden hoses, automobile upholstery and many other applications (ATSDR, 2002). The non-occupational exposure to phthalates poses high risks due to its wide use in a wide range of consumable products. Post exposure, phthalates are rapidly hydrolyzed to their respective monoesters, that is usually further bio transformed and excreted in the urine and feces (Barr et al., 2003). Phthalates also displayed *in vitro* and *in vivo* toxicity (mutagenicity, developmental toxicity and reproductive impairment among others (Bang et al., 2011).

Some compounds were exclusively found in a single sample. For examples, cyclic octaatomic sulfur, identified in sample B, is a microbiological activity indicator which is the result of organic

matter degradation in waste leachates. This could be the result of bacteria is using hydrogen sulfide (H_2S) instead of water as an electron donor in a primitive photosynthesis-like process. The lack of sulfur containing compound in other samples could be due attributed to its consumption during Organic Matter (OM) degradation, whereas OM degradation is still occurring in sample B which can be classified as freshly produced waste leachates (Badoil and Benanou, 2009). Nicotine, identified in sample E, is a substance present in tobacco. Pentacosane, identified in sample E, is a naturally occurring compound and constituent of the waxes (Peris-Vicente et al., 2006) (Belge et al., 2014).

The only compound that exists in the five samples is DEHP. DEHP has wide applications as plasticizer in PVC to make the final product soft and malleable. Plasticizers poses significant risks due to their ability to migrate from the parent material and leach out into the environment. The breakdown products of DHP have endocrine disruptors properties in addition to displaying higher toxicity than DEHP itself. DEHP is resistant to microbial biodegradation due to the positioning of the two ester groups to one another, as well as the branching on the side chains. The US EPA has expressed significant concerns about phthalates due to their toxicity and environmental risks(Erythropel et al., 2014).

Chemical analyses were performed on leachate samples collected from the five sites and several parameters were monitored including pH, Pb, Cd, Cr, Cu, Al, Mn, Fe, Zn, As, K, Na, and Ni, NO_3^-, SO_4^{2-}, PO_4^{3-}, water hardness, Cl^-, Ca, Mg, conductivity, turbidity, TOC, TC , IC (Inorganic Carbon), TDS, FS and VS in Table 2. Due to the lack of standards data for metals values in leachates, we compared our data to those cited in Lebanon in year 2002 (El-Fadel et al., 2002), in Norway (Slomczyñska and Slomczyñski, 2004) and USA (Johansen and Carlson, 1976) and the aquatic TRVs for the Ecological Risk Assessment (ERA) (Division, 1999). Nitrate concentration

ranged between 2 mg/L and 101 mg/L with highest value measured in sample D (treated leachates) which also showed the lowest levels of TOC (2 mg/L). Sample C (untreated waste sample) by contrast had a high value of TOC (278 mg/L) and exhibited very low nitrate levels (2 mg/L). The nitrate range was comparable to the levels previously reported in Lebanon except for sample D (treated leachates) which was 10 times higher the reported values in 2002. This can be attributed to nitrate actively oxidizing the water organic constituent in high TOC content, hence being present in reduced form, whereas its oxidized form prevails when the TOC content is low such as in the treated sample D (Zhang et al., 2016). The TOC range is within the Norway range and below the one previously found in Lebanon. In the assessed samples phosphate levels ranged between 8 mg/L and 50 mg/L, which are within the range of the previous study done in Lebanon. Water hardness, as calcium and magnesium, were significantly higher than those previously reported. This could be due higher alkalinity of the previously reported samples which renders dissolution of calcium and magnesium carbonate more favorable and increases the total hardness. Figure 2 portrays the variation of metals within sampling sites and includes the TRV values of aquatic organisms for comparison. This assessment helps in projecting the long-range health risk potential of the different components assessed here. As leachates are generated by solid waste land disposal sites, contamination of ground and surface waters may occur through the process of bioaccumulation and bio magnification. The health effects from leachate are not limited to drinking water as it can penetrate skin, get into the food chain leading to metals accumulating in aquatic organisms. (James, 1977). From the analysis of the data of this study (Table 2 and Figures 2), 60 % of sampling sites had Mn levels above those of TRV (120 µg/L), particularly sample B was higher by 44-fold; 80 % of sites had Al levels far beyond the TRV value (87 µg/L) reaching at sampling site A levels higher by 73-fold. In addition, 83 % of the samples had Cu levels higher than TRV (6.54 µg/L), reaching

448 at sample site C 30-fold higher, and 80 % of sampling sites had Zn levels higher than TRV (120
449 μg/L), with highest at site B by 7-fold. Nevertheless, all sites had higher levels of, Ni, Pb and Cd
450 than their TRV values of 87.71 μg/L, 1.32 μg/L, and 0.66 μg/L respectively. Ni at site B was only
451 2-fold higher than the TRV value, while at site A Pb was 12-fold higher and Cd at site B was 27-
452 fold higher. Regarding As, all sites were lower than TRV (190 μg/L). The speciation of these
453 metals and their geochemistry was examined. The association of Cu with organic constituents was
454 highly evident upon comparing Cu levels of sample site C (199.8 μg/L) with high TOC and those
455 levels of Cu in sample D (64.28 μg/L) with low TOC. This can be attributed to the stability of Cu
456 organic complexes (Shaheen and Rinklebe, 2014). The levels of Cr were much higher in sample
457 C (untreated sample) than sample D (treated sample). A most probable explanation arises from the
458 reducing media of untreated sample C rendering Cr(III) a dominant form which along with basic
459 pH (8.12) leaves Cr as suspended $Cr(OH)_3$ particles. It is important to mention here that all samples
460 were tested without filtration. An association of Cd with inorganic carbon (carbonates) was
461 evidenced in comparing sample C with high inorganic carbon (304 mg/L) and high Cd content
462 with those of site D (43 mg/L of carbonate) and low Cd content (Table 2). The strong association
463 of Cd to could be the result of similarities in the ionic radius of Cd (0.97 Å) and Ca (0.99Å). This
464 means that the Cd has the potential to enter the calcite crystal as camouflaged element and co-
465 precipitate with carbonate (Korfali and Jurdi, 2011) (Korfali, 2010).
466 The higher levels of Zn in treated sample (D) than untreated (C) can be explained by the association
467 of Zn with inorganic carbon (Korfali and Davies, 2004) in sample D which under acidic pH (pH=
468 6.34), the precipitated $ZnCO_3$ can readily dissolve and increase the level of Zn. To compare all
469 measured parameters among the different samples, the statistical t-paired test was performed using
470 the statistical SigmaStat Package. The statistical test was run to observe the parameters in samples.

Results indicated a statistical significant difference in measured parameters for sample C and D (P= 0.006). Also, statistical significant differences existed between sample B and D (P <0.001), A and D (P =0.024), E and D (P= 0.011).

The chemical analysis results discussed in the previous sections clearly indicated the presence of many chemical and metal compounds from various origins in the municipal waste leachates. The complex interaction between the various chemicals identified in the leachates makes it virtually impossible to establish the true toxicity and genotoxicity of the leachate liquids from individual chemicals detected. This complexity stems from the interactions between the different chemicals and the potential additive, synergistic and even antagonistic effects that can result from such cocktails as previously described (Khalil and Winder, 2008).

4.2. Leachates cytotoxicity assessment

Cytotoxicity analysis of the various leachate samples using single cell culture and serial dilution of the samples (as measured by the MTS assay) was presented in Figure 3. The data in Figure 3 showed that the viability changed, as the leachates concentration increased from 0.313 to 80 %. In sample A the viability significantly decreased after 2 h. from 95 to 67 % and after 24 h. from 78 to 9 %. Sample B the viability significantly decreased after 2 h. from 99 to 66 % and after 24 h. from 86 to 22 %. Sample C viability also significantly decreased from 100 to 32 %, while sample D viability decreased from 99 to 93 %. Sample E the viability decreased after 2 h. from 100 to 48 % and after 24 h. from 100 to 10 %. The data also indicated that 24 h. post leachates exposures we witnessed statistically significant differences in damages between exposed and control cells (p <0.05). The data clearly indicated the significant toxic potential of the undiluted leachates with only one exception the leachates coming from Tripoli dumpsite post biological treatment which proved less toxic than other leachates. This finding agrees with other researchers that investigated

contaminated soils from municipal dumps (Swati et al., 2017) and established loss of toxicity with higher dilutions. The data also indicated a dose dependent significant increase in cell viability upon dilution which could be correlated to dilution of chemical constituents in the leachate samples. The observed cell death at higher leachates concentration could be the result of oxidative stress leading to apoptosis and necrosis as previously reported by a number of researchers (Alabi et al., 2013) (Talorete et al., 2008). Therefore the proposed cellular model using cell cultures and MTS cytotoxicity assay (measuring cellular mitochondrial activity) (Mosmann, 1983) represented a useful approach to measure and characterize landfill leachates toxicity with more sensitivity achieved if cells are exposed 24 h. to the leachates prior to toxicological assessment. (Swati et al., 2017) (Alimba et al., 2016).

4.3. Leachates mutagenic potential

The mutagenicity potential of chemicals identified in leachates was also reported by a number of researchers using the comet assay methodology (Singh et al., 2007) (Noz et al., 1996; Collins, 2004; Deguchi et al., 2007; Gajski et al., 2012; Alimba et al., 2016). No literature could be found on the genotoxic potential of municipal waste leachates from Lebanon. The results reported in Table 4 consisted of calculated TMI. TMI is the tail DNA content of cells multiplied by the tail length and divided by 1000.

The comet assay was used to establish the genotoxic potential of leachates samples as presented in Table 3 and Figure 4. The tail moment TM analysis clearly indicated significant damages ($p < 0.05$) comparable to our positive control ($KMnO_4$) with the only exception being sample D the biologically treated sample from Tripoli landfill where lower DNA damages were observed. This agrees with other researchers that pointed out the important role of biological treatment and how

untreated leachates is characterized by higher genotoxicity by comparison to treated leachates (Widziewicz et al., 2012) (Brkanac Sandra et al., 2014).

The cytotoxicity data together with the DNA damage data reported were used to rank the sites from Most to least toxic (Table 4). The ranking of the site samples was similar although the DNA damage assessment appeared to be more sensitive in detecting early damage levels.

Table 4: Leachates sample ranking from most to least toxic using the MTS and Comet assays.

MTS assay results ranking 24 h. post exposure ranking (based on LD_{50}) (Most toxic to least toxic)	**Comet Assay Tail DNA Content Ranking (Most to least damage)**
1. Sample B	1. Sample A
2. Sample A	2. Sample B
3. Sample E	3. Positive control ($KMnO_4$)
4. Sample C	4. Sample E
5. Sample C	5. Sample C
6. Sample D	6. Sample D
7. Negative control (HBSS)	7. Negative control (HBSS)

4.4. Leachates assessment using the mouse model

The cytotoxicity data presented in this paper clearly indicated significant levels of cellular and DNA damages in cells exposed to leachates. Unfortunately the generated toxicity profiles reported using cellular systems does not always translate to animal model as reported by a number of researchers (Jakasa and Kezic, 2008) (Nesslany, 2017). Therefore we selected an animal model the BALB/c mice model as a biological indicator as previously reported by many researchers (Farombi et al., 2012) (Bakare et al., 2003) to assess the impacts of leachates exposure *in vivo* and to further validate the *in vitro* results.

The mice drinking water was substituted with diluted and pure leachates for a period of 4 weeks and data reported in Figure 5. The average organ weight of the kidneys in samples A, B, C and D was 0.17 grams while in controls 0.15 grams, that of the liver in the four samples between 0.76 and 0.93 grams while in controls 0.81 grams, and that of the spleen in the four samples between 0.11 and 0.15 grams while in controls 0.18 grams. Therefore we concluded that no significant differences in the weight of the mice between exposed and controls could be observed. Furthermore, kidney spleen and liver harvested from euthanized mice at the end of the exposure period did not show any significant differences in weight by comparison to controls. These observations were not in agreement with other studies on mice (Bakare et al., 2003) were it was determined that weight loss could be observed after leachates exposure. This discrepancy may be attributed to the subtle differences in leachate composition between landfill sites.

The enzyme activities averages in samples A, B, C and D were measured. ALP of liver ranged between 100 and 257 %, the ALP of blood between 109 and 160 %, the GPT of liver between 62 and 98 %, the GPT of blood between 89 and 100 %, the GOT of liver between 102 and 160 %, and the GOT of blood between 514 and 772 %. We reported significant increases in ALP levels in the livers (homogenized liver samples) upon leachates exposures in sample B, C and D. We also reported significant increases in liver GOT levels in samples A and B. No significant changes in GPT levels could be reported in all the samples. The observed significant expression of GOT and ALP levels in the livers of exposed mice is a clear indication of changes in liver function because activity of this enzyme has been reported as a good diagnostic tool for the damages in human liver (Fernandes et al., 2011) (Limdi and Hyde, 2003). The level of enzymatic activity was also measured in the mice blood (ALP, GOT and GPT) (Figure 6). The data indicated significant expression of GOT levels in all samples. This is a very clear indication of acute liver damage being

triggered by the leachates exposure over 4 weeks. This elevation in blood levels of GOT indicates a possible disease of the heart, liver or muscle (Babb, 1973).

Further investigation of leachates toxicity in mice was also undertaken at the DNA levels measuring the DNA damages in isolated cells from various organs of the exposed mice (Figure 7). The data presented in Figure 7 clearly indicated significant DNA damages in all cells isolated from these organs exposed to all the leachate samples. This is in agreement with many publications correlating DNA damage in bone marrow and blood in exposed mice to leachates in other countries (Siddique et al., 2005; Chandra et al., 2006) (Tewari et al., 2006).

5- Conclusion

This study investigated the potential risks posed by municipal waste leachates in Lebanon using GC/MS, chemical analysis and toxicological testing. The fate of the majority of leachates was the Mediterranean Sea. The findings of this research clearly indicated the presence of numerous organic compounds such as oleamide, hentriacontane, eicosane, tributylamine, phtalic acid derivatives, nicotine and pentacosane. The presence of DEHP in almost all samples along with other organic compounds can pose potential health risk to humans and aquatic species. This finding together with high levels of nitrates, phosphates, heavy metals and high levels of Mn, Cr, Ni, Cd and others is very alarming given the lack of efficient biological leachate treatment methods in Lebanon. Some of these identified substances posed significant health and carcinogenic risks and the levels measured were in most cases way above acceptable levels in published drinking water and US EPA guidelines. The leachates biological toxicity analysis using *in vitro* and *in vivo* assays showed the hazardous nature of the leachates especially in undiluted form in triggering acute toxicity and reactions in exposed cells and mice. Leachates were shown to decimate cell viability

in addition to triggering significant DNA damages in human derived cell cultures and mice cells. The *in vivo* data also indicated significant short-term damages in the blood and organs such as the liver which was coupled with significant damages at the DNA levels in exposed animals. These findings clearly indicated the high public health risks posed by the leachates and the urgent need to establish better national management of biological treatment and disposal of these leachates. The *in vitro* and *in vivo* data also agreed on the significant damages triggered at the molecular levels which can lead to cancer onset upon exposure.

5- Conflict of interest

The authors declare that here is no conflict of interest regarding the publication of this paper**.**

6- Acknowledgments

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