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Review

Human Cell Culture, a Pertinent In Vitro Model to Evaluate the Toxicity of Landfill Leachate/Sewage Sludge. A Review

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Abstract: Both landfill leachate and sewage sludge are complex mixtures of many potentially toxic substances in unknown and unpredictable amounts and concentrations. Both types of matrices can pose a risk to human health and the functioning of ecosystems if released into the environment. Therefore, constant monitoring of the toxicity of these mixtures is necessary. However, traditional methods of analysis of sewage sludge/landfill leachate are mainly based on physicochemical studies that do not fully reflect the effects of these mixtures on living organisms. For this purpose, research based on biological models, including mammalian, mainly human, cells is recommended and increasingly implemented. A variety of cytotoxicity tests, based on various metabolic transformations in living cells, are a very useful tool in landfill leachate/sewage sludge toxicology studies. This paper reviews the methods used in the study of the cytotoxicity of environmental matrices and the cell lines used in these studies as biological models.

Keywords: cytotoxicity; human/mammalian cell lines; landfill leachate; sewage sludge; in vitro models

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1. Introduction

Waste management is one of the key elements in the development of countries in the light of overall population growth, rapid urbanization as well as the increased rate of waste generation per capita [1,2]. Landfilling is still the most common option in the world, although it is also one of the oldest ways to eliminate waste [3–5]. Incineration is amongst the most common ways of processing wastes before landfilling. Landfilled ashes leaching through the soils exhibit hazardous potential. However, waste incineration systems emit toxic pollutants that affect human health and the environment. It is argued that newer waste incineration technologies are cleaner and have a lower environmental impact. This does not change the fact that pollutants are still produced, and adverse health effects on people living in the vicinity of the waste incineration plant, including cancer and reproductive disorders, have been described in the literature [6–8]. As a result of landfilling, waste undergoes various physico-chemical and biological processes, resulting in the formation of highly contaminated sewage called landfill leachate (LL). The composition of such a mixture depends mainly on the type of waste in the landfill, its humidity, water infiltration, degree of degradation and storage technology. However, according to Christensen et al. (2001), most LL contains some common categories of pollutants, such as heavy metals, dissolved organic matter, inorganic macronutrients, organic xenobiotics and compounds with an endocrine-disrupting potential. Due to the possibility of LL migration to the soil, as well as surface and groundwater around the landfill, they are considered an important source of environmental pollution, posing a serious threat to human and animal life and health [9]. A mixture of chemicals that is a LL of often unknown chemical composition can adversely affect ecosystems and public health when the leachate reaches soil and waters [10–12]. This type of pollution is especially common in developing countries, where there is still a shortage of specially designed landfills and leachate treatment plants [13–15]. The most frequently used methods of treating leachate from landfills in landfill leachate

treatment plants include biological methods based on aerobic or anaerobic processes, membrane technologies, physicochemical methods and electrochemical methods [16–18]. The high content of ammonium nitrogen and other highly toxic compounds inhibit the biological treatment process and reduce the susceptibility to the treatment of leachate with conventional processes. Biological treatment leads to leachate sludge in the landfill, which is characterized by a high organic matter content. This, in turn, prompts several studies to be carried out, including its potential toxicity, before it is used for various purposes, e.g., agricultural [19].

The huge amount of sewage sludge (SS) produced means that alternative applications are sought for them, for example, in agricultural areas. However, it should be remembered that these wastes may also contain large amounts of toxic compounds that are capable of bioaccumulation in living organisms, e.g., heavy metals, polycyclic aromatic hydrocarbons (PAHs), dioxins, etc. These compounds and their mixtures can be hazardous to the environment, life and human health. Some organic pollutants are degraded in the sludge treatment steps, but heavy metals may still be present in the sludge and not undergo degradation processes. They can permanently pollute the environment, especially the soil in which they settle, but also interact with other components of ecosystems, such as the atmosphere, groundwater and surface waters [20]. The toxic components of SS cause various toxicological effects, including endocrine disorders, neurotoxicity, genotoxicity and cytotoxicity, as well as changes at the ecosystem level [21,22].

It should be also mentioned what routes of exposure a human can be impacted by the chemicals present in LL and SS. One of the most important situations is accidental ingestion of, e.g., contaminated water. As a result of such exposure, LL/SS compounds may affect the gastrointestinal tract but also the circulatory system. Literature data also indicate the possibility of accidental exposure in an uncontrolled dumpsite, and in a such situation, skin is a secondary target for LL/SS compounds. Dermal absorption of contaminated soil particles may be especially dangerous for dumpsite and wastewater treatment plant workers. Additionally, migration of leachate through the soil to groundwater and then to water bodies such as ponds and lakes used for recreational purposes cannot be excluded. Then, exposure to potentially toxic substances occurs also through the skin. In wastewater treatment plants and landfills, hazardous chemicals may also float in the form of gases and solid particles in the air reaching the human respiratory tract and the skin of people working there or living nearby [23–25].

Among human health consequences caused by contact with LL and SS, both acute and chronic diseases could be included. Inhaling gases emitted from landfills may cause a loss of coordination, nausea, vomiting, and their high concentration may even cause death [26]. Gases such as nitrogen dioxide and sulfur dioxide, when inhaled or swallowed by humans, cause symptoms such as irritation of the nose and throat, bronchospasm, and respiratory infections, especially in patients with asthma. These effects may induce asthma attacks in patients with asthma [27]. People, as a result of contact with LL, whether in gaseous or liquid form, are at risk of worsening lung function, asthma, ataxia, paralysis, vomiting, emphysema and lung cancer if heavy metals are inhaled or ingested. Diseases such as high blood pressure and anemia are caused by heavy metal contamination [28]. In addition, heavy metals in large proportions affect the nervous system, resulting in neurotoxicity leading to neuropathy with symptoms such as memory impairment, sleep disturbance, anger, fatigue, head tremors, blurred vision and slurred speech. They can also cause kidney damage, such as initial tubular dysfunction, risk of kidney stone formation, and kidney cancer [29].

Typically, chemical and physico-chemical analyses are being used in order to assess the hazard and risk associated with LL, focusing primarily on heavy metals and organic compounds with toxic, estrogenic and carcinogenic potential, even in trace amounts [30–32]. However, since such studies have proved insufficient, integrated approaches to the toxicity assessment of environmental matrices have been proposed, in which chemical analyzes are supported by toxicological assessment using bioassays on model organisms as well as

in vitro systems [33]. The main benefit of biological tests is their ability to respond to all chemical and biological agents present, which is a measure of the overall toxicity of the test matrix [34]. The correct selection of the biological model for in vitro analyzes determines the obtained biological response, which will take into account the bioavailability and interactions of all factors causing additive, synergistic or antagonistic effects in the selected model [35]. The frequency of using biological tests based on human cell lines in vitro has undoubtedly increased in recent years in an undoubtedly noticeable manner. They are also used more often to assess the risks associated with landfills [36,37]. The objectives of this review are to make a state-of-the-art biological test allowing the evaluation of the toxicity for the human of the chemical compounds released by the LL/SS. For this purpose, a literature review was carried out focusing on new reports in the field of cell-based bioassays used to estimate the potential risk posed by LL and SS for humans. More than 70 papers published since 1984 were analyzed with focusing mainly on the last 10 years articles considering mainly human-based models.

2. Methods for Determining Cell Viability

In the determination of the toxicity of the particular compound or mixture of compounds in vitro methods are being used that allow for the measurement of the changes associated with metabolic disorders of human or animal cells caused by the tested substance. In the case of LL/SS, the preparation of samples before adding them to the cell culture is a specific process. Samples after collecting them from wastewater treatment plants (SS) and/or waste landfills (LL) should be obligatorily filtered through membranes with 0.45 µm porosity. Subsequently, the resulting aqueous extracts from each sample should be filtered using 0.22 µm porosity syringe filters to prevent microbiological contamination of cell lines used in the research [38]. As there are many different organic compounds in the chemical composition of both LL and SS, some of them, especially those with high molecular weight or associated with microorganisms, may remain on the filter membrane. Therefore, it should be taken into account that the tested SS and LL filtrates have a changed chemical composition compared to the raw LL/SS. Therefore, in terms of chemical composition and physico-chemical parameters, not only raw samples but also the tested filtrates should be analyzed. Changes in the metabolic activity of cells are analyzed in cultures treated with the test substance, and the reference system is usually the culture without the compound. Since LL and SS are mixtures of many different chemical compounds, they are usually added to a cell culture within a certain range of percentages. Usually, it is between 0.1% and 20% or 30%. Measurements are usually performed using colorimetric, fluorescent, luminescent and isotope techniques. The most frequently used measure of the toxic activity of a test substance is the determination of the IC50 coefficient, which is 50% inhibition of cell growth and proliferation. A large proportion of cell viability assays are based on measuring the integrity of cell membranes using a variety of techniques. Some of the tests are related to the measurement of the metabolic activity of cells, thus indicating the potential ability of cells to show normal metabolic activity and consequently to proliferation and growth processes. In order to analyze the cytotoxicity of test substances, the protein or DNA content of the cell culture is also measured. The quality of the obtained test result is influenced by many factors. These are, among others: the type and stability of the cell line used, selection of the type of cell line for the analyzed substance, selection of the test technique, validation of the test protocol. Regarding human cell lines as a model for cytotoxicity studies, a possible way of exposure to LL/SS should be considered, e.g., when accidental ingestion is considered, cells from the gastrointestinal tract should be used (Caco-2, DLD-1, HepG-2 cell lines) [38–41].

Tests based on the measurement of cell membrane integrity include the Trypan blue staining test, which stains dead cells blue. This dye is not able to penetrate the cell membrane of a living cell, but after cell death, when the membrane is permanently damaged, and the potential between the outer and inner side of the membrane disappears, it can

penetrate the cytoplasm and nucleus. This group of compounds also includes lysamine green, eosin Y and erythrosine B [42].

The determination of cell viability may also be based on the use of compounds that freely penetrate the cell membrane of living cells but in an esterified form. Their decomposition and de-esterification cause them to precipitate in the cytoplasm, and due to their poor solubility in the pH of the cell, they accumulate. As a result of the decomposition of such compounds, products are formed that cannot penetrate the intact cell membrane. These are, e.g., calcein AM and fluorescein diacetate (Figures 1–3) [43].

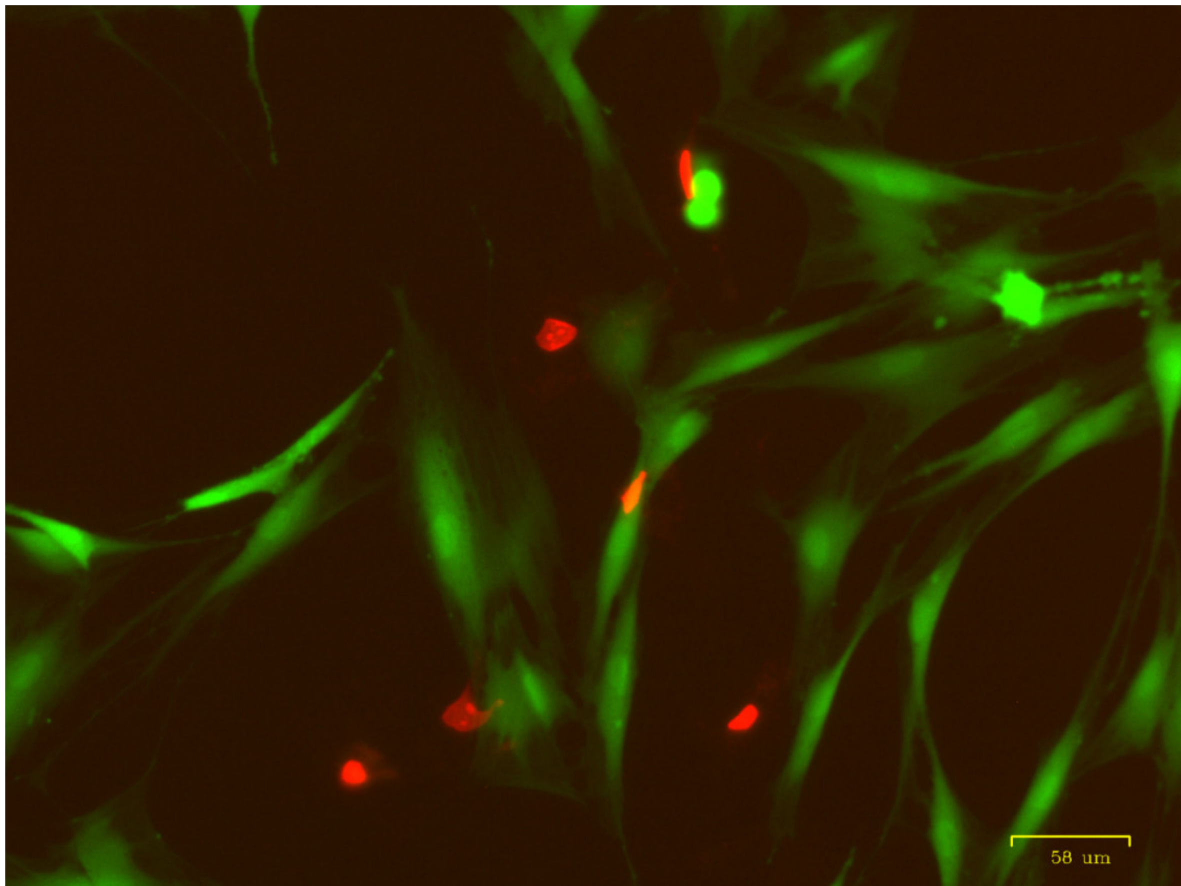

Figure 1. Fibroblast cells stained with Calcein-AM and propidium iodide. The cells were photographed using an Olympus IX83 fluorescent microscope with SC180 camera with the Cell Sens Dimension 1.17 program. Living cells are stained with green color, and dead cells are characterized by red nuclei.

Fluorescein in a cell that has an intact cell membrane and active esterases emits green light in response to blue light excitation. If the cell membrane is damaged, then fluorescein diffuses into the environment surrounding the cell, and the cell itself becomes invisible. If dead cells are also to be visualized, then dyes accumulating in damaged cells through their binding with nucleic acids should be used. These are, for example, propidium iodide, acridine orange and ethidium bromide (Figures 1–3). They intercalate between base pairs in the DNA double helix. To enable simultaneous imaging of dead and living cells, two different dyes should be used, e.g., ethidium bromide and fluorescein diacetate [44].

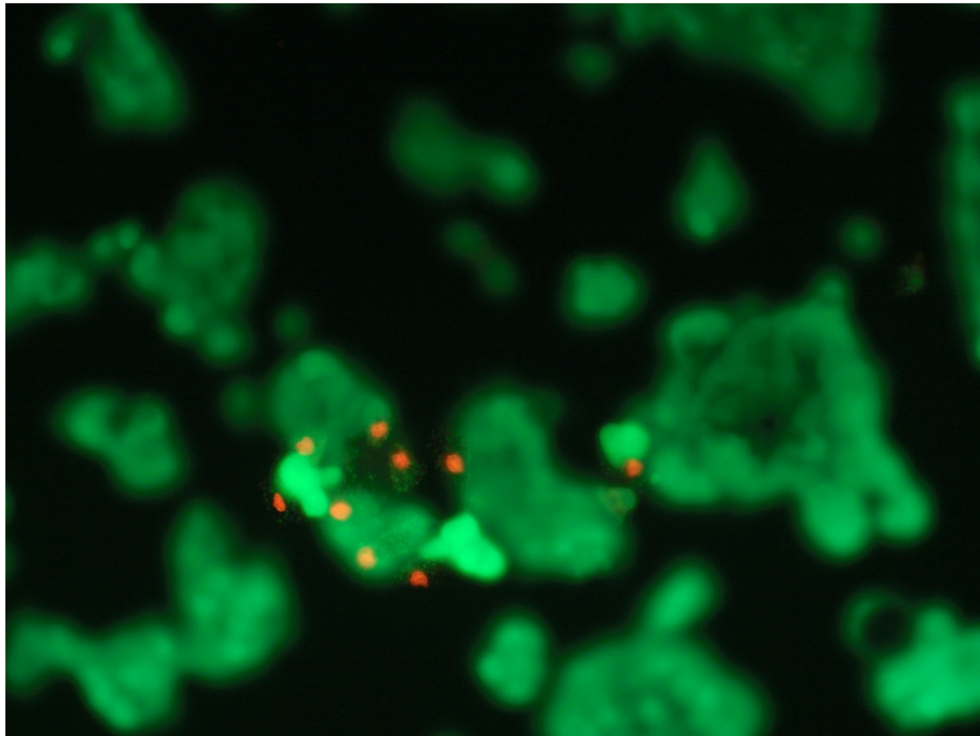

Figure 2. ZR-75-1 cells stained with Calcein-AM and propidium iodide. The cells were photographed using an Olympus IX83 fluorescent microscope with SC180 camera with the Cell Sens Dimension 1.17 program. Living cells are stained with green color, and dead cells are characterized by red nuclei.

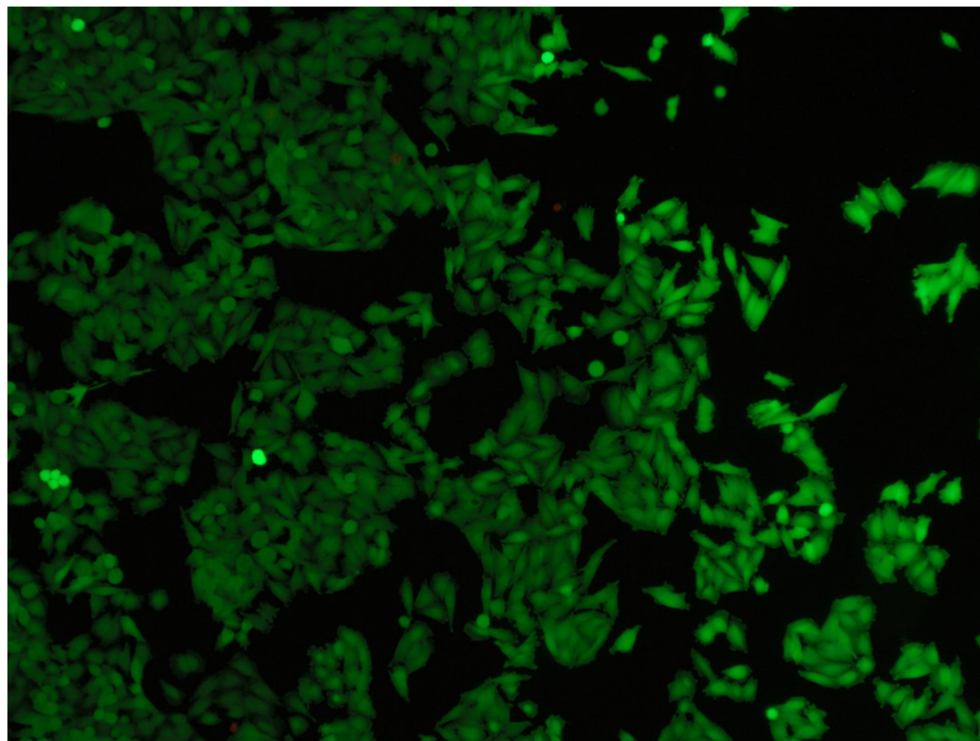

Figure 3. Melanoma cells (A-375) stained with Calcein-AM and propidium iodide. The cells were photographed using an Olympus IX83 fluorescent microscope with SC180 camera with the Cell Sens Dimension 1.17 program. Living cells are stained with green color, and dead cells are characterized by red nuclei.

Very commonly used cytotoxicity tests for test substances are based on determining the functions of individual cell organelles, e.g., mitochondria or lysosomes. An example of such a dye used to determine cell viability based on the integrity level of cell membranes is neutral red, NR. This dye is able to penetrate the membrane of living cells and accumulates in lysosomes. Red-stained viable cells are observed in the hemocytometer chamber. For the measurement of NR, one can also use a spectrophotometer and measure the absorbance after dissolving the dye in 1% acetone solution with 30% ethanol [45]. Another option is to measure the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the culture medium. If this enzyme is present in the medium, it means that cell membranes have been damaged. This test is based on the measuring of the activity of LDH catalyzing the reaction in which NADH is reduced to β-nicotinamide adenine dinucleotide. Given that NADH peaks in absorbance at 340 nm and the rate of decline in NADH levels can be measured, the results obtained can be used to determine LDH activity. A prerequisite for this is the determination of the levels of NADH and pyruvate. [46,47]. Another test that is also based on demonstrating the existence or non-integrity of cell membranes is the NAG test. If the cells in the culture are damaged, e.g., by the action of a xenobiotic, then N-acetyl-beta-D-glucosaminidase can be detected in the culture medium. It is an enzyme normally found in the lysosomes of living cells, and therefore, its determination may be used in studies of the cytotoxicity of compounds or mixtures of compounds [48]. One of the best developed and most used tests also today is the MTT test. It examines the activity of succinate dehydrogenase present in the mitochondria of living, metabolically active cells. This enzyme converts the soluble tetrazolium salt into its reduced form, which is insoluble and forms violet to dark navy-blue crystals visible in the cells. The crystals are dissolved in DMSO or isopropanol/HCl to give a violet-colored solution, the color intensity of which is measured spectrophotometrically. Formazan crystals are not formed in dead cells. The color intensity of the resulting solution is directly proportional to the number of viable cells [49]. Analysis of protein content in cells can also be used to determine the toxicity of selected compounds or mixtures thereof. An example of such a test is the Sulphorhodamine Test (SRB). It is based on the electrostatic binding of sulphorhodamine to cellular proteins at an appropriate pH. The reaction depends on the qualitative composition of the amino acids in the sample and is carried out after fixing the cells with trichloroacetic acid. The amount of protein in cells is directly proportional to the number of cells in the sample [50]. Most of the above-mentioned methods are based mainly on spectrophotometric measurements. However, it should be remembered that they are not always possible to apply. An example may be the situation when the tested compounds or their mixtures, such as LL or SS, contain in their composition compounds that interact with the components of the medium, causing an incorrect absorbance reading. Sometimes even trace amounts of metals can cause distortions in the results of the spectrophotometer. Then, cytotoxicity tests based on, for example, luminescence measurements, which exclude the above-mentioned methodological problems, are recommended.

In order to determine the cytotoxicity of selected compounds or their mixtures, methods based on the efficiency of energy processes taking place in the mitochondria are also used. An example of such a method is the measurement of the ATP content in cells, assuming a constant amount of ATP, characteristic for a given cell line, under certain culture conditions. An increase or decrease in the number of living cells is directly correlated with the amount of synthesized ATP, i.e., a change in its content is associated with a change in the number of cells. These methods are usually based on bioluminescent measurements. The oxidation reaction of luciferin to oxyluciferin is catalyzed by luciferase and proceeds with the participation of one ATP molecule. The intensity of the light emitted in this process is measured by luminescence and expressed in photons or relative luminescence units. In the analysis of the amount of ATP in the tested sample, the dependence of the intensity of luminescence on the amount of ATP and the number of cells is used [51]. The AB test (alamarBlue) is the test based on the measurements of the fluorescence produced by resorufin produced in the redox conversion process of resazurin. The results obtained

in such a measurement make it possible to evaluate the enzymatic activity of the oxidation and reduction processes [52].

It is also worth mentioning that the analysis of DNA damage in a cell relatively quickly provides information about potential toxic environmental samples. One of the most common methods of measuring DNA damage from exposure to potentially toxic compounds is single cell gel electrophoresis, known as comet assay. It is a relatively simple, fast and sensitive method combining the techniques of biochemical detection of DNA double-strand breaks, DNA single-strand breaks, alkali labile sites and DNA–DNA and DNA–protein cross-links with a typical cytotoxicity test. The amount of denatured DNA fragments migrating from the nucleus during electrophoresis is determined. The rate of migration of DNA in an agarose gel is proportional to the degree of its damage. The image of a cell obtained as a result of the test with a distinct "head" and "tail" containing damaged, migrating DNA resembles a comet, hence the name-comet assay [53].

As previously mentioned, both landfill leachate and sewage sludge are potential sources of endocrine-disrupting chemicals [54]. These are compounds that interact with natural hormones, causing disturbances in the functioning of the human reproductive and immune systems. Exposure to this type of compound is associated with an increased incidence of breast cancer worldwide. In order to analyze the estrogenic potential of the matrices in question, the E-Screen test was introduced, in which various breast cancer cell lines with different expression of estrogen receptors are used. These include MLVN, MELN, MCF-7 BUS, MELP and T47D-Bluc. Cells are cultured in a steroid-free medium to which specific amounts of LL/SS are added, and usually, after 6 days, the number of cells is compared with cultures exposed to 17beta-estradiol, which is a positive control [55].

Depending on what mechanism we want to investigate, this type of test should be selected. If the main goal is to demonstrate the potential estrogenic properties of LL/SS, then the best choice is the E-Screen test and the use of transfected breast cancer cell lines such as MELN. On the other hand, if the toxic effects of LL/SS are to be manifested by DNA damage, then comet assay is a good choice. On the other hand, when it is only necessary to demonstrate potential cytotoxicity, we can choose from a very large set of tests based on spectrophotometric, fluorescent and luminescent analyzes based mainly on cell viability.

3. The Use of Selected Human Cell Lines in Studies of the Basal Toxicity, Genotoxicity, Cytotoxicity and an Estrogenic Potential of LL and SS

In vitro cytotoxicity methods are among the preferred methods, especially when compared to in vivo methods. This is related to the possibility of avoiding many complex and problematic logistic procedures, reducing costs, shortening the duration of the research and bypassing the ethical issues inherent in animal testing.

The ability to quickly detect the potentially cytotoxic, genotoxic and estrogenic properties of chemicals and their mixtures, such as LL or SS, by in vitro testing is excellent. In vitro tests are an effective screening tool suitable for a large number of samples. In addition, in vitro tests can be used to analyze environmental samples for specific mechanisms of action, which may indicate the presence of compounds with a specific mode of action in the cell in the mixture. However, extrapolating the results of in vitro tests to whole organisms and even ecosystems has many unknowns because in vitro tests are not intended for modeling or assessing the systemic effects of a given substance. They are useful for determining biological activity, interaction potentials and mechanisms of action but do not take into account metabolic processes in organisms and interactions at the tissue level [1,33,56,57]. Frazier (1993) developed a description of the in vitro model consisting of three basic components: the biological model, the effect and the protocol. The biological model is a system in which the effects of a potentially toxic substance are tested. The second component is the effect of the substance or its mixture in a biological system after exposure to a cell model. The third component is the protocol as a set of experimental conditions (temperature, time, reagents and equipment) necessary to repeatedly measure the effects in the model [24,58].

A possible route of exposure of humans to LL/SS could be accidental ingestion of contaminated water. In this case, the best choice for studying the potential toxicity of LL/SS towards humans is using gastrointestinal tract cell lines, such as DLD-1, Caco-2 or HepG2. The key organ that is primarily exposed to the influence of various environmental pollutants is the liver, which plays a detoxifying role in the body. It is exposed to the components of the LL after it has been accidentally swallowed, for example, from LL contaminated water. Accidental ingestion is listed as the main route of exposure to contaminated environmental matrices by the USEPA risk assessment guidelines. The most common human liver cell line is HepG2. It is a well-known biological model of the liver that maintains the morphological features of parenchymal liver cells and has active xenobiotic-metabolizing enzymes involved in their detoxification. It is one of the most widely used lines in drug metabolism and toxicology studies. They have been used in the cytotoxicity analysis of environmental matrices since 2011, and until 2019, they were one of the most frequently used lines [59–61].

The next possibility of human exposure to LL/SS is breast tissue, which could be affected by LL/SS components after ingestion, making the circulatory system an important potential way of delivering toxic substances to the whole organism. Due to the presence of many substances from the group of endocrine-active compounds in SS and LL, human cell lines representing various types of breast cancer are used as biological research models [54,62]. These include, among others, cell lines such as MCF-7, MDA-MB-231, ZR-75-1, MVLN, T47D, MELN and MELP. They represent both estrogen-dependent and estrogen-independent types of breast tumors [55,63–65]. One of the most frequently used methods is above mentioned E-Screen test, in which the proliferation activity of cells of a selected breast cancer line is compared under the influence of tested LL/SS samples, and then it is compared with the proliferation activity of cells of the same line exposed to 17-β-estradiol. The MCF-7 line is the breast cancer line most commonly used in this type of toxicological research [66].

Other models of human cells are also used to study the cytotoxicity of complex environmental matrices, especially those to which skin is exposed. These include skin-derived cell lines such as fibroblasts (NHDF) and keratinocytes (HaCaT). The skin is a very important organ through which soil microparticles contaminated with leachate can be absorbed by, for example, accidental exposure to an uncontrolled landfill. Human exposure may also occur through recreational activities, ingestion of contaminated water or aquatic biological resources. Therefore, it seems necessary to analyze the potential toxicity of the LL/SS to skin cells. It also seems important to check whether the analyzed matrices have a carcinogenic effect, promoting the growth and development of skin cancer cells. Then, the melanoma cell line, e.g., A-375, seems to be a suitable research model [23,53,67,68].

As it was mentioned before, the circulatory system could be also an important potential target for humans after accidental ingestion of contaminated water. Peripheral blood cell lines are a large group of cell lines used in the study of the cytotoxicity of environmental matrices. These include lymphocytes isolated from patients' whole blood. They are exposed to contaminants from environmental matrices also as a result of accidental ingestion and are considered cells to show early signs associated with adverse health effects after exposure to the contaminants [69,70].

Other cell lines very commonly used in LL and SS cytotoxicity studies are the human HOS osteosarcoma line, Jurkat lymphoma cells, CHO line, LN229 glioblastoma and mouse fibroblast line NIH/3T3 [71–74].

In Table 1, different human cell lines and tests are compiled with obtained results.

Table 1. A list of papers with different cell lines and viability/toxicity/genotoxicity/estrogenic potential tests and their results.

Viability/Toxicity/Genotoxicity/ Endocrine Disrupting Test	Cell Line Used	Results	Reference
MTS assay, adenyl kinase release	HepG2	cytotoxic in low doses (2.5-5%)	[60]
MTT assay, alkaline comet assay	HepG2	EC50 value ranging from 11.58 to 20.44%	[75]
fluorescence microscopy (acridine orange/ethidium bromide)	lymphocytes	cytotoxic effect of raw leachate; treated samples showed no cytotoxic effect	[76]
MTT assay cell scratch damage	MCF-7	Phenols extracts from landfill leachate could slow down the rate of migration of cells	[63]
MTT assay	LN-229	no cytotoxic activity of sewage sludge filtrates	[71]
comet assay	NHDF (normal human dermal fibroblasts), Me45	decrease in LL toxicity after treatment	[53]
MTT assay CellTiter-Glo™ 2.0 Assay	A-375 fibroblasts	landfill leachate cytotoxic to fibroblasts, no cytotoxic effect in A-375	[23]
MTT assay	A-375 fibroblasts	sewage sludge cytotoxic to fibroblasts, no cytotoxic effect in A-375	[67]

4. Conclusions

Both landfill leachate and sewage sludge are complex environmental matrices with potentially high toxicity. They can pose a threat to the environment, human life and health because they can penetrate into groundwater and soil, contaminating them, even at large distances from direct storage sites. This is especially true of illegal, uncontrolled landfills, which are usually not provided with adequate systems for treating and discharging hazardous leachate. The most widely used methods for testing the toxicity of such mixtures are based on chemical analyzes and biological models using microorganisms such as bacteria, algae, small invertebrates, fish and plants. However, only the models of mammalian, and especially human cell lines, are able to partially reflect the effects of toxins on the human body. In all cited studies, the leachate was cytotoxic, genotoxic, carcinogenic, mutagenic and estrogenic. Research conducted on human cell models is very useful in analyzing especially cancerogenic and/or estrogenic potential of complex environmental matrices such as LL/SS, but it should be underlined that they also have some limitations. The obtained results cannot be directly extrapolated to whole organisms because using single-cell lines does not allow studying the relationships and metabolic changes that occur in tissues and entire organisms. However, the use of several different biological tests in conjunction with chemical analyses and the study of the physical properties of the leachate/wastewater will allow for the development of an integrated strategy for the toxicological analyzes of complex environmental matrices.

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