Chapter 2

Analysis of the suitability of currently used methods for assessing the toxicity of contaminants found in sewage sludge and biowaste

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2.1. INTRODUCTION

As a result of constant growth of the amount of sewage sludge and biowaste, managing these substances has become a significant ecological problem. The annual production of dry matter of sewage sludge in the European Union is more than 10.96 million tons (He et al., 2014). For many years, sewage sludge and biowaste were mostly stored, burned or composted. Another way of neutralizing sewage sludge and biowaste, which is more economical and often more environment-friendly, is using them as a fertilizer on agricultural soils (Latare et al., 2014). Sewage sludge and biowaste may be rich in organic matter as well as macro and micro elements, so they can serve as an alternative for fertilizers and successfully increase the dry matter yield of various crops (Singh and Agrawal, 2008).

Decisions on how to manage the sludge largely depend on the knowledge of chemical and biological hazards identified in the sludge. Both municipal and industrial sewage can be contaminated with chemicals dangerous for human life and health, which cannot be neutralized as part of processes used in sewage treatment plants and thus concentrate in the produced sewage sludge. One group of such contaminants is refraction compounds (which are hard to decompose or cannot be decomposed biochemically). Among them, especially dangerous are toxic contaminants, causing physiological disorders in plant and animal organisms, and in higher doses, even death. Toxic substances are i.a., heavy metals (e.g., arsenic, copper, lead, cadmium, mercury, zinc, chromium and nickel), polycyclic aromatic hydrocarbons (PAHs), aromatic amines, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDDs/Fs), pesticides, pharmaceuticals and many others (Kapanen et al., 2013). These contaminations have at least one of the following characteristics: carcinogenic, mutagenic or

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teratogenic (causing defects in the development of an embrio or a fetus) activity and the ability to bioaccumulate in the food chain of humans and animals.

The need to manage sewage sludge and reintroduce all the substances occurring in it to the ecosystem generates the need to carry out quantitative and qualitative tests of contamination in sewage and sewage sludge. The development of chemical analysis methods allows us to identify most chemical compounds present in the sludge. However, identification is not enough to answer the following questions:

- What influence can the specific substance (with the specific concentration) have on plant and animal organisms living in the ecosystem?
- How can a substance with a specific concentration affect the human organism directly and indirectly?

Bioindication is the method of assessment of the environmental status that allows us to learn the total toxicity caused to the ecosystem by all the harmful substances taken together. This method involves the use of living organisms present in the environment, called bioindicators, indicator organisms or stenobionts, which have a very narrow range of ecological tolerance to a certain agent (or agents). For example, the presence of crayfish in a lake proves the lack or low concentrations of toxic substances, and the presence of bees in an ecosystem proves the lack or low concentrations of pesticides in the vegetation. When analyzing the total toxicity, we should remember mutual influences of the identified compounds. Different groups of substances present in the environment may interact and thus affect the results of toxicity tests. The interaction may be positive (synergy effect, enhancing the toxicity) or negative (antagonistic activity, reducing the toxicity). The toxicity of the ecosystem as a whole is not equal to the sum of individual toxicities of each substance present in it.

The composition of sewage sludge or biowaste does not provide all the information about its hazardous potential. It is not enough to precisely assess the potential hazard caused by its use for natural or industrial purposes. Objective assessment is possible thanks to toxicological tests, including bioindication assays. Toxicity is a property of a substance causing disorders in biological functions or the death of cells, organs or entire organisms. The effects of toxic activity result from chemical or physicochemical reactions between the toxic substance and the biological system of the organism. Toxicity may be acute or chronic. Acute toxicity tests show the effects substances occurring i.a., in surface waters, bottom sediments, soils, sewage, sewage sludge or biowaste have on organisms. Chronic (long-term) toxicity tests provide information concerning the negative effects of substances on individuals and populations in the conditions of prolonged activity.

The results of toxicity tests obtained for a specific substance using the same species and in the same conditions are not always repeatable. This may be caused by various adaptation factors of organisms, leading to differences in the effects and toxicity levels of particular substances. Therefore, different concepts and measures are used when assessing toxicity. The measure of toxic influence on the organism is the amount of chemical substance causing (or not causing) a biological effect expressed as the proportion of organisms responding to that amount. This value is

provided in weight units with reference to the body mass or surface area, and less frequently, to the time of exposure to the toxic substance.

The end point which is the easiest to observe in toxicity tests is the death of the organism as a result of exposure to a lethal dose. In order to be able to compare and evaluate the effects of the tested substances, the concept of lethal dose (LD) has been introduced. LD is the amount of a substance that causes death after one-time administration. Laboratory tests usually determine LD_{50}, i.e., the dose of the toxic substance causing the death of 50 out of 100 tested organisms. For gaseous substances, lethal concentration (LC) is determined. The minimum dose (dosis minima, DM) is also used to refer to the lowest amount of the substance causing the first observable effects of toxic substance activity. Another measure is the effect concentration (EC), causing any changes in tested organisms, e.g., inhibition of growth or biochemical processes. The most frequently determined value is EC_{50}, referring to the concentration of the toxic substance that inhibits the physiological process by 50%. The result is provided with reference to the experiment duration (Walker et al., 2005).

2.2. TOXICITY TESTS

Many sewage treatment plants in Europe are not able to produce environment-friendly sewage sludge that could be used i.a., to fertilize soils (Mininni et al., 2015). The mobility of chemical compounds present in sludge and biowaste and the degree of releasing them to soils depend on a number of factors, such as the pH and chemical composition of the soil, organic matter present in it, redox potential and metal speciation (Malara and Oleszczuk, 2013). The contact of hazardous sewage sludge and biowaste with soils may cause the accumulation of dangerous substances in soil and their transfer to the food chain at different trophy levels (Pathak et al., 2009). Hence, it is necessary to use simple, quick and cheap but also accurate and sensitive analytical strategies of assessing the toxic environmental impact of chemical compounds present in sludge and biowaste. Therefore, a number of biological tests of acute toxicity have been developed to determine the levels of toxicity of those compounds for water and land organisms. The tests involve the use of microorganisms, plants, invertebrates or fish. Currently, several dozen bioindication methods are used to evaluate the impact of potentially hazardous substances on various parts of the ecosystem. These include a number of methods to assess the effects of toxic influences of substances present in sewage sludge and biowaste.

Methods combining chemical analysis of hazardous compounds and biological toxicity tests have proved to be the most effective in identifying the main toxic substances in sludge and biowaste. The use of toxicity identification evaluation (TIE) procedures allows us to obtain information of the most toxic contaminants present in the studied medium and help determine their total share in the general toxicity of the tested sample (Ferraz et al., 2017).

2.2.1. BACTERIAL ASSAYS

Assays based on testing the effects of metabolic activity of microorganisms exposed to substances present in sewage sludge or biowaste are very popular. A number of methods make use of bacteria to test toxicity. The assessment of chemical stress is usually quick, cheap and accurate, which makes it possible to apply that procedure to many samples at a time. There are some standard, widely used tests based on growth inhibition, e.g., the *Pseudomonas* growth inhibition test (Flockton et al., 2019) or the activated sludge test (Friedrichs et al., 2017). Among the most common are *Aliivibrio fischeri* or *Photobacterium phosphoreum* bioluminescence inhibition assays. *Aliivibrio fischeri* is a Gram-negative, rod-shaped bacterium found globally in marine environments. It is bioluminescent and is mostly found in symbiosis with various marine animals. The bacterial enzyme luciferase allows *Aliivibrio fischeri* bacteria to naturally emit light as a result of the following reaction:

$$FMNH_2 + O_2 + R - CO - H \rightarrow FMN + R - COOH + H_2O + LIGHT$$

The intensity of the generated light is proportional to the metabolic state of the cell. Stress conditions caused by the presence of toxic substances have a negative impact on metabolism, thus slowing down the cell activity and reducing the intensity of the generated light.

Since those bacteria live in a water environment, an aqueous solution containing the substances accumulated in sludge or biowaste must be prepared before the experiment. Through the comparison of intensity of light emitted by the bacteria in the tested sample and in the control sample (containing physiological saline solution) we can calculate the percentage of inhibition (I%) using the formula:

$$I\% = [1 - (\text{sample light/control light})] \times 100$$

The result of the assay is the EC_{50} value, i.e., effective concentration of the toxic substance causing 50% reduction of luminescence (Table 2.1). The EC_{50} values obtained after 15 minutes are converted into toxicity units (TU [g/L]) using the formula:

$$TU = [1 / EC_{50}] \times 100$$

A number of classification scales (e.g. by Persoone, Liebmann) have been developed to determine the class of toxicity. One of them is the system proposed by Persoone, with the following classes depending on the obtained TU value:

- class 0 – TU = 0 – no toxicity
- class 1 – 0 < TU < 1 – no significant toxicity
- class 2 – 1 < TU < 10 – significant toxicity
- class 3 – 10 < TU < 100 – high acute toxicity
- class 4 – TU > 100 – very high toxicity

Table 2.1

Toxicity values obtained by *Vibrio fischeri* expressed as 50% bioluminescence inhibition (EC_{50}) and toxicity units (TU) (Farré and Barcelo, 2003)

Substance	EC_{50}, µg/mL	TU
Acetaminophenol	173	0.58
Alcohol ethoxylate: $C_{10}EO_x$	3.25	30.76
Alcohol ethoxylate: $C_{12}EO_x$	0.55	182
Benzene sulphonate	1223	0.082
2-Chlorophenol	34.82	2.87
4-Chlorophenol	21.21	4.71
Dichlofluamid	0.136	735.3
2,4-Dichlorophenol	2.85	35.09
Endosulfan	5.63	17.8
Fluorene	4.1	24.2
Ibuprofen	12.1	8.26
Methomyl	1005.89	0.099
Naproxen	21.2	4.76
16 PAHs	0.19	520
Phenantrene	0.13	797
Phenol	7.99	12.5
Polyethylene glycol	127.4	0.79
Sea-nine (antifoulant)	0.0584	1712
2-(thiocyanomethylthio) benzothiazol: TCMTB	0.0268	3731

The luminescence assay has many advantages. It is quick, sensitive, accurate and repeatable. The simplicity of the assay means it can be used to test various environmental samples, including sewage, sewage sludge extracts and biowaste. An ISO standard including high repeatability and simplicity of experiment has been established for the assay based on bioluminescence inhibition (Escher et al., 2017; Di Nica et al., 2017; Rubinos et al., 2014).

2.2.2. BIOSENSORS

Biosensors are analytical devices that can be used to assess the toxicity of the tested sample. They convert biological signals into measurable ones. The output signal is a biological element of the microoorganism, e.g., enzyme (Nguyen et al., 2019) or DNA (Kavita, 2017), which is detected and connected to a converter that converts it to a measurable signal (Table 2.2). Toxicity tests based on bacterial biosensors contain immobilized live bacteria cells. For example, a test based on the inhibition of conductivity of a polymer covered with agarose involves *Saccharomyces cerevisiae* impregnated on an agarose layer. Biosensors can detect various biological variables, e.g., changes in bacterial UV absorption. Other tests are based on changes in cellular respiration, checking the number of electrons

produced in this process with the use of a pair of electrodes (a carbon electrode and an Ag/AgCl reference electrode) and an amperometric sensor (Table 2.2).

Table 2.2

Principal transduction systems used in biosensors (Turdean, 2011)

Transduction system	Measurement	Parameters
Electrical	conductometry	conductance
Electrochemical	amperometry potentiometry	current voltage at zero current
Piezoelectric	mass-quartz crystal microbalances mass-surface acoustic waves	mass velocity and so forth
Optical	fotometry fotometry refractometry	luminescence fluorescence refractive index
Thermal	calorimetry	temperature

The CellSense system based on electrolysis can be used to test turbid samples or even suspensions. The measurements are not disturbed by the sample's turbidity, which is definitely an advantage of that technique (Table 2.3).

Table 2.3

Toxicity values obtained using the CellSense biosensor either with *Pseudomonas putida* and *Escherichia coli* expressed in 50% bioluminescence inhibition (EC_{50}) and toxicity units (TUs) (Farré and Barcelo, 2003)

Substance	*Pseudomonas putida*		*Escherichia coli*	
	EC_{50}, µg/mL	TU	EC_{50}, µg/mL	TU
Alcohol ethoxylate: $C_{10}EO_x$ Alcohol ethoxylate: $C_{12}EO_x$	75 69	1.33 1.45	92 596	1.08 0.168
2-Chlorophenol	296	0.34	250	0.4
4-Chlorophenol	239	0.42	201	0.498
2,4-Dichlorophenol	247	0.4	393	0.254
Endosulfan	3.38	29.6	ni	ni
Pentachlorophenol	320	0.31	0.037	2703
Polyethylene glycol 2,4,6-Trichlorophenol	33 256	3.03 0.39	400 0.67	0.25 149.2

ni – not investigated

A disadvantage is the impossibility to test the toxicity of samples including substances that can precipitate on the electrode or remove its bacterial film. This means that many substances, including aggressive solvents, are excluded. Each measurement must be preceded by the test of electrochemical activity of the sample. Sensitivity to some substances and low repeatability of results are the main

disadvantages of biosensors based on a system of electrode pairs (Table 2.4) (Farré and Barcelo, 2003; Malhotra and Turner, 2003; Turdean, 2011).

Table 2.4

Advantages and disadvantages of a whole cell-based biosensor (Turdean, 2011)

Advantages	Disadvantages
– more sensitive and detailed than chemical methods, – produces real-time data and can be applied in field work or in situ analysis, – fast, less expensive, and less intensive labour, – cheaper to use because the active biological component does not have be isolated and because microorganisms are living, unlimited quantities can be prepared relatively inexpensive, – react only to the available fraction of metal ions, – does not involve specialized training and the bulky, fragile equipment	– short lifetime, – conditions (reagents, incubation time, pH, temperature) can effects the biosensor performances, – limited of selectivity, – lack of genetic stability

2.2.3. TESTS USING INVERTEBRATES

The presence of earthworms is one of the most important factors contributing to soil loosening and fertilizing. They live in all types of soil. There are approx. 800 worms in various stages of development in 1 m^3 of soil. These organisms fragment and mineralize organic matter, changing the structure and chemical composition of soil. Therefore, they are very susceptible to the activity of all substances present in the soil, especially the toxic ones, which can be introduced to the soil with hazardous sewage sludge or biowaste (Babić et al., 2016; Kinney et al., 2012). *Eisenia fetida* is a species used in biohumus production and in waste disposal processes. It can live up to 15 years long: the longest out of the several hundred described earthworm species, whose life cycle is on average 4 times shorter. This species reproduces quickly and is highly sensitive to many toxic substances occurring in soils. Because of its advantages, it is often used in ecotoxicological tests. *Eisenia fetida* is one of the best organisms for ecotoxicological assays carried out with the use of soils and solid waste for which OECD and ISO toxicity standards have been established (Nahmani et al., 2007). Experiments using earthworms can be used to assess both acute and chronic toxicity. Chronic toxicity caused by the conditions of long-term exposure to the tested substance or group of substances present in soil or sludge is most often tested. The expected measurable and directly observable effect of such experiments is usually the reduction in biomass growth, increased mortality, color changes, mobility problems and problems with reproduction (a lower number of cocoons)

(Babić et al., 2016; Molina et al., 2013; Xing et al., 2014). The toxic effect can be assessed, not only through direct observation but also, i.a., through measurements of morphological changes, multixenobiotic resistance mechanism (MXR) activity or lipid peroxidation levels (the biological process of lipid oxidation leading to the formation of lipid peroxides). The standardized MXR test is based on the measurement of model fluorescent dye concentration in earthworm bodies. Even short-time exposure to a toxic substance results in observable MXR inhibition, whose degree is proportional to the amount of the stressor (Babić et al., 2016).

Apart from direct observation of the influence of hazardous substances on the development of *Eisenia fetida*, histopathological assays (microscopic identification of lesions occurring in organisms' cells and tissues) are also performed. Feeding on organic substances present in soil, earthworms absorb them via their alimentary system, which leads to their exposure to direct contact with toxic substances occurring in it. Organisms exposed to the activity of substances present in soils for a longer period of time also absorb them indirectly, via their thin, semipermeable epidermis. Histopathological assays allow i.a., for the identification of physiological changes in earthworms' organs and tissues and determine the impact of hazardous substances on the thickness of body walls, mid-gut epidermis injury, and the area of gut resorption (the process of substance transpiration through surfaces) (Babić et al., 2016; Christofoletti et al., 2012).

2.3. SUMMARY

The discussed methods of ecotoxicological assessment of contamination in sewage sludge and biowaste are commonly used all over the world. All the assays involve living organisms, but none of them is universal enough to be undeniably better than the others. The main reasons for this are the kind of microorganisms used and the limitations of particular methods. Each microorganism used in the tests has a different specificity. It may be very sensitive to some groups of toxic substances but resistant to others, even if only to a small extent. The compounds may be widely occurring in the environment, very rare, or even completely new, not yet tested for their negative effect on the ecosystem. Therefore, in order to determine the degree of toxicity of a substance and its dangerous dose, we should perform tests involving various organisms living in the given environment. Different species display different levels of resistance to the same substance. Bacteria and invertebrates are organisms that develop most frequently in sewage sludge and biowaste which can enrich soils in valuable organic and mineral matter, so the available assays most often involve some of these.

There are at least several automated devices on the global market whose operation is based on the analysis of intensity of light generated by luminescent bacteria, e.g., Tox-Alert from Merck, Microtox from Azur Environmental or LUMIStox from Beckman Instruments (Farré and Barcelo, 2003). Figure 2.1 shows the Microtox system used for tests at the Czestochowa University of Technology.

Notwithstanding all its advantages, the test using *Vibrio fischeri* has some limitations. The maximum methanol concentration tolerated by luminescent bacteria is 10%. Moreover, these bacteria are marine microorganisms, so in order for the assay to be accurate, filtration in a saline solution is necessary before each test. The salinity of the sample reduces the solubility of some organic substances, which causes the turbidity of the studied solution. Despite these limitations, assays based on the analysis of bacteria bioluminescence have for many years been among the quickest and most effective tests to assess toxic influence on the environment.

The main advantages of biosensors are the possibility of mass production, online readings, quick reaction and simplicity of application. Methods used to create biosensors allow us to select the best test species for the assessment of toxic effect caused by particular substances occurring in the sample. The most important is the proper choice of microorganisms that will be sensitive enough to detect the expected groups of hazardous substances. Another factor that plays a role is whether the microorganisms can be immobilized. Furthermore, the appropriate measurable biological signal should be selected. As already mentioned (Chapter 2.3), due to the processes occurring in the device, not all substances can be tested using this technique (Farré and Barcelo, 2003).

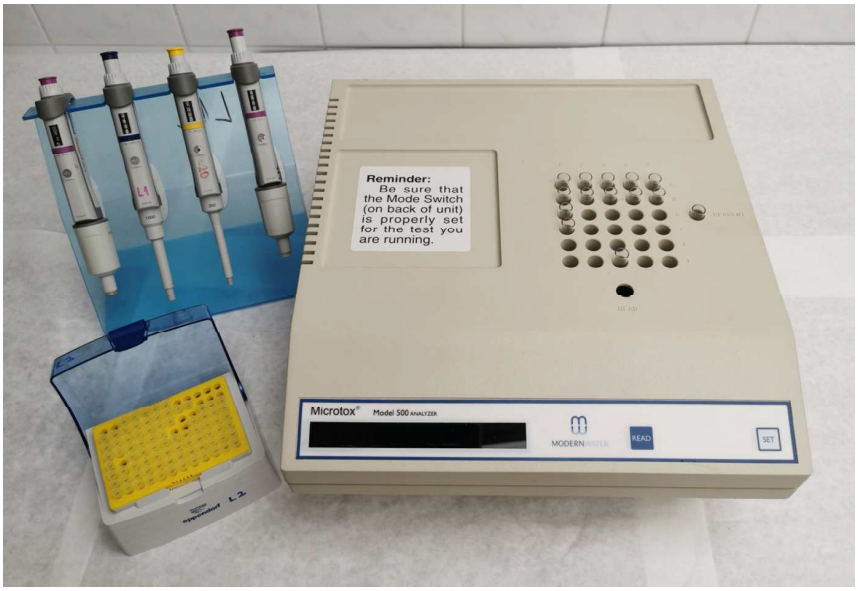

Fig. 2.1. The Microtox M500 system used for tests at the Czestochowa University of Technology

Because they allow for testing various physiological factors and perform morphological, histopathological, and even behavioral analyses, biological assays using earthworms allow to identify precisely (much better than do tests using microorganisms) the mechanisms of activity of hazardous chemical substances

present in sludge and biowaste. In addition, the life cycle of *Eisenia fetida* makes it possible to perform short- and long-term tests of acute and chronic toxicity. However, such tests have a lower automation level, are more expensive, more labor-intensive, and take much longer than do experiments with the use of microorganisms (Babić et al., 2016).

Each of the discussed methods of assessing the toxic effect has its limitations, which make it impossible to obtain reliable results for some groups of substances. Still, they are all quick, simple and cheap techniques. The use of various test species provides exhaustive information on environmental hazard, and the combination of these methods with chemical analysis of the tested substances is a good approach to the identification of the most toxic fractions and hazardous compounds present in sludge and biowaste.

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