BEHAVIOR OF PLANTS AND MICROORGANISMS IN THE PRESENCE OF INORGANIC POLLUTANTS

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Abstract. *Rapid industrialization along with the use of modern practices in agriculture led to the discharge into the environment of various pollutants from which metals and metalloids are the most identified. The presence of metals in soil affects soil fertility, the growth and development of plants, and finally causing different negative effects on human health such respiratory problems, damage to endocrine and nervous systems and other dysfunction. Over time, various techniques have been used to remove pollutants from contaminated media, but most of these techniques are expensive, produce secondary contaminants and modify the structure and fertility of the soil. Thus, in recent years considerable attention has been accorded to biological methods. Plants and microorganisms may exhibit different behavior and degree of tolerance in the presence of metals.*

Keywords: ecotoxicological tests, metals and metalloid, indicators of toxicity, phytotoxicity tests, tolerance

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1. Introduction

Soil contamination with potentially toxic elements (PTEs) (metals or non-metals) represent a global concern because their presence is endangering both the environment and human health [1]. The group of metals includes both essential elements for normal metabolic processes named micronutrients (Fe, Mn, Cu, Zn, Ni, Mo), which in excessive amounts are more harmful to plants compared to animals, and non-essential elements (As, Hg, Pb, or Cd) which are very harmful to humans and animals affecting the growth and development of plants even at low concentrations [2, 3].

According to the U.S. Environmental Protection Agency (USEPA), Hg, Cr, Pb, Ar, Co, Cd, Co, Zn, Ni, Be, Mn and Sn are the most toxic metals [4]. The

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presence of metals in soil affects the structure and degree of fertility. In case of plants, metals inhibit their normal growth by suppressing various processes such as photosynthesis, respiration, carbohydrate metabolism and water transport. It has also been reported that the accumulation of metals in plants reduced the plant lengths due to functional interference in the absorption and distribution of important nutrients and minerals such as Ca, Mg, Fe, P and K. Metals present negative effects on human health, affecting the reproductive system, inducing nutritional deficiencies, endocrine disorders, tumors and other chronic diseases [5, 6] (Fig. 1).

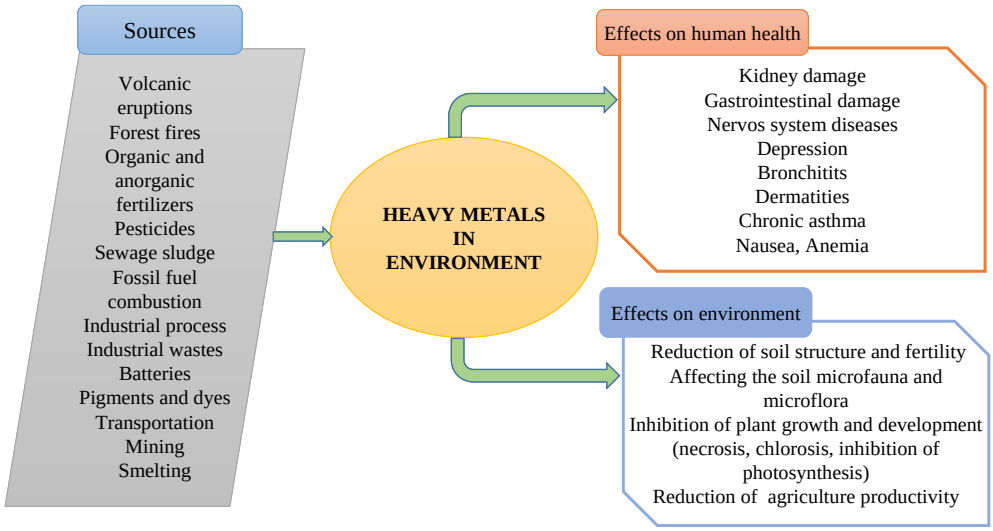


Fig. 1. Source of metals and their effects on human and environment [5-7]

Plants and microorganisms can tolerate different concentrations of pollutants, and may further adopt different resistance strategies against metals toxicity. Phytotoxicity test is one of the simplest methods in biomonitoring the environment being the first step in understanding the behavior of plants in the presence of pollutants. Based on these tests, the preliminary effects of pollutant toxicity on plants and the tolerance of plants to a certain level of contamination are determined [8]. Ecotoxicological tests are also important tools for assessing the toxic potential of pollutant for different microorganism species [9].

2. Behavior of plants in presence of metals

Plants may react differently in the presence of pollutants and also may have different tolerance to them [10]. Non-tolerant plant species are affected by the presence of metals, inducing a wide range of effects on plant cellular activities (photosynthesis, respiration, mineral nutrition, membrane structure and properties, and also gene expression). Although all the plants can be affected by metals in

high concentrations, some species are quite tolerant to low quantities, especially the plants from *Brassicaceae* family. Of these, *Brassica juncea* has been identified as a crop with high biomass, with the ability to uptake and accumulate metals such as Cd, Co, Ni, Zn, Pb and Se [11].

The effects of pollutants on the early stages of plant growth were widely studied. In this regard, seed germination is an important indicator in determining the effects of pollutant toxicity on plants. Such studies are effective in identifying the proper pollutant-tolerant plants [12]. The tolerance of plant in the presence of pollutants can be defined as the ability to survive in an environment that is toxic to other plants, although the term is frequently used in the literature as suggesting the changes (effects) that may occur on plants in response to pollutant toxicity [13]. Thus, the following aspects must be taken into account in assessing the degree of plant tolerance to heavy metals [13]:

1. Tolerance to metal is specific;
2. Different degrees of tolerance can be recognized in a population of a certain species;
3. Tolerance to metal is inherited.

Exposure to heavy metals triggers a wide range of physiological and biochemical changes, and plants need to develop and/or adopt different strategies in order to protect against the negative effects of heavy metals. Plants respond to external stimuli, including the toxicity of heavy metals through several mechanisms. These include detecting external stress stimuli, signal transduction and signal transmission in the cell, and initiating appropriate measures to reduce adverse effects. At the level of the whole plant, it is difficult to measure changes after exposure of plants to metals. However, monitoring of early responses, such as oxidative stress, transcriptomic and proteomic changes, or enzymatic activity are used to evaluate the changes that may occur after exposure of plant to pollutants [10]. For example, Tamás et al. [14] reported that the signs of Cd and Hg toxicity on barley plants were similar to the signs of water deficiency. Similar to this, Hernandez et al. [15] reported that oxidative stress and reduced glutathione in alfalfa roots are due to exposure to heavy metals. In another study elaborated by Zhang et al. [16], it was found that seed germination and wheat seedling growth are inhibited due to the high concentration of As. Similarly, Muhammad et al. [17] reported a reduction in the length of roots of *Helianthus annuus* L. when was exposed to As concentration.

Plants can adopt various ways of detoxifying the excessive concentration of heavy metals. Detoxification can take place in either the cell wall, cell membrane or protoplasm. Also, the increase in glucose content under the influence of metal stress has led to a reduced carbon metabolism and disruption of photosynthesis reactions that ultimately lead to growth inhibition [18]. Azmat et al. [19] in their study indicated that variations in leaf anatomy and morphology of *Phaseolus*

mungo and *Lens culinaris* reflect their adaptability to environmental stress. Lead induced changes in epidermis of leaves, such as reducing the size of cells, increasing the number of stomata and trichomes (hairs or scales grown on the epidermis of a plant with a protective role), reducing the size of protective cells. Thus, the increase of trichomes and the number of stomata on the surface of the upper leaves of the mentioned plant species represent an adaptive morphological mechanism to the presence of pollutants.

1.1. Indicators of pollutant toxicity to plants

Phytotoxicity represents the ability of a compound to cause short-term or long-term adverse effects on plants. The phytotoxicity of different heavy metals differs and the order of toxicity in plants is known to be in the following order: $As^{5+}<As^{3+}<Cr^{6+}<Co^{2+}<Ni^{2+}<Cu^{2+}<Ti^{+}<Hg^{2+}<Cd^{2+}<Ag^{+}$. The higher concentration of these metals in plant cells, the more physiological, biochemical and cellular changes that lead to severe effects on plants [20].

Germination tests of plant seeds in Petri dishes using filter papers moistened with a solution of pollutant is the most common method used to assess the phytotoxicity of contaminants on plants [21]. Seed germination is divided into 4 phases (imbibation, radicle emergence, radicle elongation, seedling growth) as illustrated in Fig. 2.

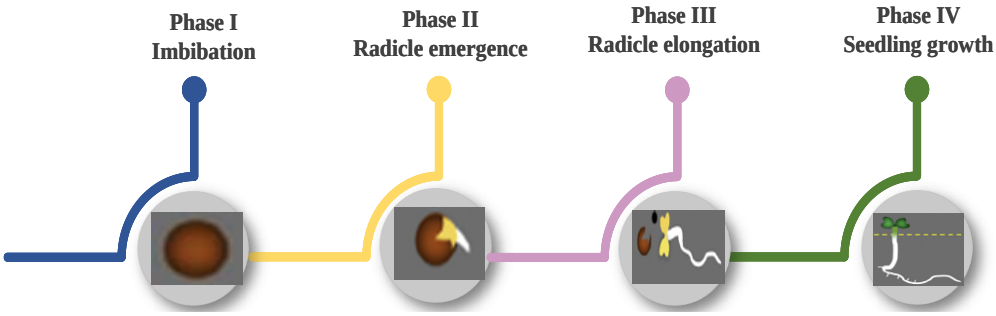


Fig. 2. Stages of seed germination [22]

Phytotoxicity tests are simple methods that requires minimal costs, the seeds can be easily purchased, they remain viable for a while time and the germination period is relatively short [23].

1. *Germination degree (GD,%).* Seed germination is influenced by both intrinsic factors (physiological and hormonal of the seed) and external factors (environmental conditions during seed development). There can be several causes that prevent seed germination and plant growth, so metal ions can affect the activation of essential enzymes that are needed in the germination process. Metals such as Cd, Cu and Pb have been shown to inhibit the activities of these enzymes

[17]. A seed is considered germinated when it is over 2 mm long [22, 24, 25]. *Brassica* species are widespread, their seeds having a medium size, which germinates quickly [26]. The data that can be obtained from phytotoxicity tests take into account the degree of germination of seeds compared to the control sample. The degree of germination (GD%) is the most used index and represents the ratio between the number of seeds germinated in the pollutant sample and the number of seeds germinated in the control sample [22] (Eq. 1).

$$GD(\%) = \frac{Number\ of\ germinated\ seeds\ in\ metal\ concentration}{Number\ of\ germinated\ seeds\ in\ blank\ sample} \times 100 \quad (1)$$

2. *The germination index (IG)* is the ratio between the degree of seed germination in the pollutant samples and the degree of seed germination in the control sample (Eq. 2).

$$IG = \frac{GD\%_{test}}{GD\%_{blank}} \times 100 \quad (2)$$

where: $GD\%_{test}$- degree of seed germination in sample with pollutant; $GD\%_{blank}$- degree of seed germination in blank sample.

3. Tolerance index or elongation rate for roots and shoots (Er, %). In order to evaluate the phytotoxic effects, the lengths of the roots and shoots of plants subjected to different concentrations of pollutants are measured. The tolerance index or elongation rate is calculated by relating the lengths of the roots/shoots of the pollutant samples to the lengths of the roots/shoots of the blank sample [27, 28] (Eq. 3).

$$E_r = \frac{L_{test}}{L_{blank}} \times 100 \quad (3)$$

where: L_{blank} - mean length of roots/shoots of the sample with pollutant (mm); L_{media}- mean length of roots /shoots in blank (mm).

4. Toxicity index or elongation inhibition rate of roots and shoots length (EI, %). Another parameter used in order to establish the toxicity of pollutants on plants is toxicity index or elongation inhibition rate for roots and shoots (% from blank lengths) (Eq. 4) ([29]:

$$E_r = \frac{L_{blank} - L_{test}}{L_{blank}} \times 100 \quad (4)$$

5. Relative growth rates of biomass (Br, %) as a percentage from blank sample. Plant biomass production is closely related to plant growth in length, so the higher the biomass production the more successful the phytoremediation process is [30] Pollutants have negative effects on the amount of biomass, so once

absorbed by the plant, they cause a reduced biomass production of the roots, or on the whole plant development [31]. The relative biomass growth (Br, %) represents the amount of biomass obtained (green or dry) compared to the blank sample (Eq. 5):

$$Br(\%) = \frac{Biomass_{test}}{Biomass_{blank}} \times 100 \quad (5)$$

3. Behavior of microorganisms in presence of heavy metals

Bacteria were among the first life forms that appear on Earth, with nearly 6.5 million species identified on land and another 2.2 million species in the oceans [32]. The major factor that determines the toxicity of heavy metals to bacteria is probably the extent to which they enter in the cytoplasm. Metals can also be prevented from entering the cell by forming complexes or chelates with multiple metal binding agents [33]. Some of the effects of pollutants on microorganisms are related to the changes in cell surface morphology and growth behavior; cell membrane disruption; inhibition of enzyme activity; oxidative phosphorylation leading to lipid peroxidation; protein denaturation [34-37] (Table 1).

In order to establish the effects of some pollutants on microorganism species, first of all, their tolerance in the presence of pollutants is studied, followed by the analysis of pollutants retained on/in biomass to establish the bioremediation capacity. To analyze the tolerance of microorganisms to different metals and to see the effects of metals on microorganisms that are able to survive, grow and develop in the presence of pollutants, enzymatic activity represent the first indicator to study these effects. Thus, the chemical reactions that take place inside an organism are catalyzed by enzymes and any metabolic disturbance is observed at the level of enzymatic activity. The growth of microorganisms in the presence of pollutants can lead to the generation of reactive oxygen species (ROS) that cause oxidative stress in cells. Excessive accumulation of ROS and its intermediates inside cells can cause oxidative stress in the body, such as oxidation of amino acid side chains that disrupt the structure and function of proteins, leading to inactivation of enzymes, damage to the cell membrane, damage to cell division and followed by cell death [38].

Exposure to heavy metals increases the production of ROS in the cells of microorganisms which in turn triggers their defense system to prevent oxidative damage [38]. The defense mechanisms against oxidative stress induced by free radicals involve: prevention mechanisms, recovery mechanisms and antioxidant defense. The antioxidant defense system of microorganisms comprises several enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) glutathione peroxidase (GPX), and among the non-enzymatic antioxidants are glutathione (GSH) and acid ascorbic acid (CA). Glutathione is the most abundant intracellular thiol that can prevent the destruction of cells caused

by ROS, which reduces the sulfur bonds being transformed into its oxidized state, glutathione-disulfide (GSSG) [39, 40].

Table 1. Toxicity of heavy metals on microorganisms [35]

Metal	***Metal Effects***
Nickel	Affects the cell membrane, enzyme activity and causes oxidative stress
Cadmium	Denatures proteins, destroys nucleic acid, prevents cell division and transcription
Arsenic	Inactivation of enzymes
Chromium	Inhibition of growth, prolongation of the adaptation phase (lag), inhibit oxygen uptake
Copper	Interrupts cell functions, inhibits enzymatic activity
Selenium	Inhibits growth rate
Lead	Destroys nucleic acid and proteins, inhibits enzyme functions and transcription
Mercury	Denatures proteins, inhibits enzymatic activity, disrupts the cell membrane
Silver	Lysis of cells (disintegration), inhibits cell transduction and growth
Zinc	Death, lowers biomass, inhibits growth

Thus, in the first phase, the bacterial cells developed defense mechanisms to detoxify/neutralize ROS. These defense mechanisms pose the following functions [41]: *prevention of radical formation*; *elimination of radicals by reaction with antioxidants*; *destruction of ROS using the action of enzymes;* restoration of *oxidative damage; accelerating the elimination of damaged molecules; elimination of strongly damaged molecules to minimize the occurrence of possible mutations.*

Heavy metals and other pollutants lead to increased production of reactive oxygen species. The results of Khalid and Jin [42] study, showed that Zn^{2+} and Pb^{2+} lead to the production of reactive oxygen species and cause an imbalance in the cells of microorganisms. The activities of the antioxidant enzymes of the catalase, peroxidase and ascorbate peroxidase of the microorganism *Bacillus subtilis* Kh were increased when it was subjected to the concentration of 100 mg $Zn^{2+/}$L and 100 mg $Pb^{2+/}$L. The value of the catalase activity was 141% at the concentration of 100 mg Zn^{2+}/L, and the peroxidase was 260% at 100 mg Pb^{2+}/L, respectively 160% at 100 mg $Zn^{2+/}$L.

Although microbial strains have the ability to tolerate metals, to survive and increase in the presence of these pollutants, interactions with these metals can lead to metabolic or physiological changes in microorganisms. In the study elaborated by Rizvi and Khan [37], it was observed that changes in cell surface and in the growth behavior of the microorganism *Pseudomonas aeruginosa* CPSB1 increased in the presence of 400 µg Ni/mL. A reduction in cell size, irregular edges of the bacterial colony, agglomeration and aggregation of cells when exposed to different concentrations of heavy metals (400 µg Zn/mL, 400 µg Pb

/mL, 400 µg Cr/mL) were also observed compared to uniform, normal stems with smooth edges and undamaged cells of *Pseudomonas aeruginosa* CPSB1, when it is grown in the absence of metals [37]. Also the results of Chen et al. [42] study showed that the presence of cadmium in concentration of 200 mg/L altered the cell membrane of the microorganism *Enterobacter sp*. EG16. *Enterobacter asburia* tolerates nickel up to a concentration of 2000 µg/mL, and *Pantoea agglomerans* tolerates cadmium up to 3000 µg/mL, so following the SEM analysis (electron scanning microscopy), the presence of cadmium induced changes in the size and surface characteristics of microorganism cells [43].

1.2.Indicators of pollutant toxicity to microorganisms

The toxicity of heavy metals represents the ability of a metal to cause harmful effects on microorganisms and depends on the bioavailability of heavy metals and the dose absorbed.

Pollutants can cause disturbances to the biological system of microorganisms, so the parameters used to assess the toxic effects of pollutants include growth inhibition, respiratory activity, antioxidant enzymatic activity. Estimation of respiratory activity is used as the first indicator to assess the toxicity of chemicals on bacteria [9, 44].

Superoxide dismutase (SOD) activity. Superoxide dismutases (SODs) are the first bacterial enzymes involved in the detoxification of ROS, therefore they are some of the most important defense enzymes against oxidative stress [40]. SOD catalyzes the dismutation of the superoxide anion (O^{2-}) into hydrogen peroxide and molecular oxygen [45] (Eq. 6):

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (6)$$

SOD protects cells against the harmful effects of superoxide free radicals, which are one of the most toxic forms of reactive oxygen species. To calculate SOD activity, Eq. (7) is used [46]:

$$SOD\ Activity = \frac{\frac{\Delta A_{325blank} - \Delta A_{325test}}{\Delta A_{325blank}} \times 100\%}{50\%} \times 4.5 \times \frac{1}{V} \times D \quad (7)$$

where: $\Delta A325_{blank}$- self-oxidation rate in control sample; $\Delta A325_{test}$ - self-oxidation rate in the sample with pollutant; V - volume of sample (mL); D - sample dilution factor; 4.5 - final volume of the reaction mixture (mL).

Catalase activity (CAT). Oxygen peroxide (H_2O_2) is a toxic by-product of all metabolic processes that prevent cell damage, it must be converted into other less

toxic substances, and thus catalase is the only antioxidant enzyme capable of converting oxygen peroxide into water and oxygen (Eq. 8) [47]. It is found in all aerobic microorganisms, plants and animals. Like superoxide dismutase, catalase is very important in protecting cells against oxidative stress, the catalase being extremely effective, it can break down millions of molecules of hydrogen peroxide in a second [48].

$$2H_2O_2 \rightarrow 2H_2O + O_2 \ (gas\ bubbles) \tag{8}$$

Catalase activity is calculated with the equation (Eq. 9) [46].

$$CAT(U/mL) = \frac{3.45 \times df}{t \times 0.5} \tag{9}$$

where: *3.45*- decomposition of 3.45 μm hydrogen peroxide into a 3mL reaction mixture leading to a decrease in absorbance of 0.05; *df*- dilution factor; *t*-time required for the absorbance to decrease by 0.5*; 0.5*-amount of enzyme extract.

Dehydrogenase (DEH) activity. Dehydrogenases are produced by a large number of microorganisms and other living organisms [49]. Dehydrogenase activity can be used as an indicator of biological redox systems commonly used in biochemical experiments to indicate in particular cellular respiration [50].

The measurement of dehydrogenase activity is based on the quantification of TPF (triphenylformazan) produced by the reduction of TTC (triphenyltetrazolium), being usually a rapid analysis that can be obtained in a few hours. Specific dyes, such as 2,3,5-triphenyltetrazolium (TTC) which is a hydrogen acceptor, is introduced into a cell suspension, the hydrogen is transferred by dehydrogenases to this colorless compound, which is reduced to 2, 3, 5-triphenylformazane (TPF), a red compound. TPF can be quantified spectrophotometrically at a wavelength of 485 nm [46, 49]. The reaction equation is described by Eq. (10):

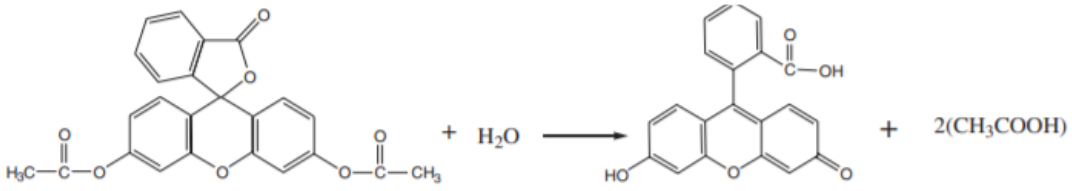
$$TTC \rightarrow \xrightarrow{+2e^- + 2H^+} TPF \tag{10}$$

The color intensity of the formazan extract obtained is determined spectrophotometrically at the wavelength of λ = 485 nm. The dehydrogenase activity expressed in μg / mL is determined using a calibration curve and the formula (Eq. 11) [46]:

$$\alpha = \frac{(Pcs - Pbs) \times V \times 100}{t} \tag{11}$$

where: α-dehydrogenase activity (μg/mL); *Pcs* - *TPF* concentration in the analyzed sample (μg/mL); Pbs - concentration of TPF in the control sample (μg/mL); *V* - volume of cell suspension taken (mL); *t* -sample incubation time (h).

Hydrolase activity (FDA-H). Hydrolases facilitate the cleavage of C-C, C-O, C-N bonds and other bonds by water. Hydrolytic enzymes break down major chemical bonds in toxic molecules and thus reduce toxicity. Hydrolases can be grouped according to the nature of the chemical bond and they cleave into amylases, phospholipases, proteases, lipases, DNases and xylanases [49] Fluorescein diacetate (FDA) can be used to measure microbial activity. Fluorescein diacetate is hydrolyzed by a number of different enzymes, such as proteases, lipases and esterases. Enzyme activity leads to hydrolytic cleavage of FDA (colorless) into fluorescein (yellow-green fluorescent). The reaction equation is described as follows (Eq. 12) [51]:



Fluorescein diacetat Fluorescein

(12)

The quantification of the enzymatic activity is performed by evaluating the color intensity of the spectrophotometer at a wavelength of 490 nm. To determine the amount of fluorescein produced in a sample Eq. (13) can be applied [46]:

$$F = \frac{(DO_P - DO_{C1} - DO_{C2})y}{G \text{ or } V} \tag{13}$$

where: *F* - the amount of fluorescein produced in either a solid / liquid sample (gram or mL); PDO - absorbance of the sample; DO_{C1} - absorbance of control sample 1; DO_{C2} - absorbance of control sample 2; *y* - the slope factor in the calibration curve; *G or V* - weight or volume of the sample to be analyzed.

Optical density (OD_{600}). Optical density (OD_{600}) provides information about the growth of microorganisms. Specifically, microbial growth correlates with the number of microorganisms in a population and reports how it changes over time. The method is based on the mode of detection of absorption and is practically determined by the portion of light passing through a sample, more precisely through a suspension of microorganisms [52]. Monitoring the change of a

microbial population over time results in a characteristic curve (Fig. 3) called the growth curve. It consists in four growth phases [52]:

- ***Phase I lag (adaptation phase)*** - After placing a few microorganisms in the culture medium (inoculation), the organism must adapt to its new environment. During this time there is no division and the density remains the same.

- ***Phase II - Log (exponential phase)*** - Once the microorganism has adapted to its new environment, it begins to reproduce. Each cell doubles in a specific time, which leads to exponential microbial growth. The log-phase is the time when microorganisms are most effective. This phase also helps to calculate growth parameters, such as doubling time and growth rate.

- ***Phase III (Stationary Phase)*** - The reproduction of microorganisms is limited by the nutrients present in the culture medium. Once the carbon source or other essential components are consumed, the microorganisms no longer divide.

- ***Phase IV decline and cell death*** - As the environmental conditions of a microbial culture worsen due to the reduction of nutrients, salts, amino acids, the population begins to die. This becomes visible through a general decrease in the density of microorganisms.

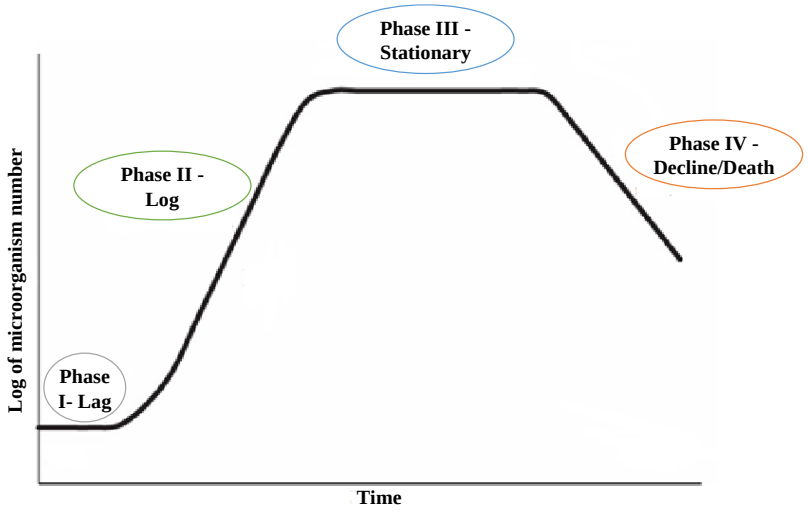


Fig. 3. Microbial growth curve [52]

The degree of cell development is calculated by (Eq. 14) [46]:

$$OD600/mL = \frac{(OD_{Tn} - OD_{To}) \times df}{V} \quad (14)$$

where: OD_{Tn} - optical density of the sample at a given time (e.g. 24 hours, 48 hours, etc.); OD_{T0} - optical density of the sample at time 0 immediately after sowing; *fd* - dilution factor; *V* - sample volume (microbial culture) subjected to analysis.

Conclusions

Contamination of environmental components with heavy metals causes negative effects on environmental and human health due to them toxicity and non-biodegradability properties, thus it is absolutely necessary to eliminate or reduce their concentrations in the environment.

Several plants may react differently in the presence of heavy metals which can be affected at the cellular, metabolic and structural levels, where the most obvious effects of toxic compounds on plants are inhibition of the seed germination and on root and shoots growth development. However, there are some species of plants that are capable to tolerate different concentrations of heavy metals, and also to accumulate high amount of heavy metals in their tissues. Also, some of microorganisms present the ability to survive at different concentrations of heavy metals and can furthereven develop defense mechanisms and also to adsorb metals in the outer layers of the cell.

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